THE ANTI-CANCER ACTIVITY OF THE NATURAL PRODUCT, FUCOIDAN, IN HAEMATOLOGICAL MALIGNANCIES

by

Farzaneh Atashrazm, BSc, MSc

Menzies Institute for Medical Research

University of Tasmania

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

University of Tasmania

November, 2015

Declaration of Originality

"This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright."

Farzaneh Atashrazm

Signed: .. .. Date: 3.11.2015

Authority of Access

This thesis may be made available for loan. Copying of any part of this thesis is prohibited for two years from the date this statement was signed; after that time limited copying and communication is permitted in accordance with the *Copyright Act 1968*.

Farzaneh Atashrazm

Signed: . ..... Date: 3.11.2015

Statement Regarding Published Work Contained in Thesis

"The publishers of the papers comprising Chapters 3 to 6 hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non published content of the thesis may be made available for loan and limited copying and communication in accordance with the *Copyright Act 1968*."

Farzaneh Atashrazm

Signed: .. .. Date: ...3.11.2015.....

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Farzaneh Atashrazm

Signed: . ..... Date: 3.11.2015

Statement of Co-authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Farzaneh Atashrazm[1] designed and carried out the experiments, analysed the data, drafted the manuscripts and completed revisions.

Ray M Lowenthal[1] designed and supervised the study and edited the manuscripts.

Gregory M Woods[1,2] designed and supervised the study, analysed the *in vivo* data and edited the manuscripts.

Adele F Holloway[2] carried out experimental design for the *in-vitro* study and edited the manuscript.

Samuel S Karpiniec[3] carried out the analysis of fucoidan composition.

Joanne L Dickinson[1] directed design and supervision of the study, analysed the data and edited the manuscripts.

[1] Menzies Institute for Medical Research, University of Tasmania
[2] School of Medicine, University of Tasmania
[3] Marinova Pty Ltd., Cambridge, Tasmania

Signed: .. Date: ...3.11.2015...

Farzaneh Atashrazm

Candidate

Signed: / Date: ...3/11/15...

A/Prof Joanne L Dickinson

Primary Supervisor

Publication arising from the period of my candidature:

Paper: Fucoidan and Cancer: A Multifunctional Molecule with Anti-Tumour Potential
Publisher: Marine Drugs; **Submitted:** 24 Feb 2015; **Accepted:** 3 Apr 2015

Paper: Fucoidan suppresses the growth of human acute promyelocytic leukaemia cells *in vitro* and *in vivo*
Publisher: Journal of Cellular Physiology; **Submitted:** 12 Jun 2015; **Accepted:** 30 Jul 2015

Paper: Fucoidan enhances the therapeutic potential of arsenic trioxide and all-trans retinoic acid in acute promyelocytic leukaemia, *in vitro* and *in vivo* **(Undergoing Peer Review)**

Data arising from this thesis has been presented at the following scientific meetings:

- Poster Presentation. 44th Annual Scientific Meeting of International Society for Experimental Haematology (Kyoto, Japan, 15-17 September 2015). Anti-tumour activity of fucoidan in acute promyelocytic leukaemia and its synergy with arsenic trioxide and ATRA *in vitro* and *in vivo*.
- Oral Presentation. 5th Congress of the International Society for Applied Phycology (ISAP) - Seaweed as anti-cancer Symposium- (Sydney, 22-27 June 2014). **Atashrazm F**, Lowenthal RM, Dickinson JL, Holloway AF, Woods G. Irhimeh M. Anti-tumour activity of fucoidan through inhibition of Erk activation in human acute promyelocytic leukaemia.
- Poster presentation: 19th Congress of European Haematology Association (Milan, Italy 2014). **Atashrazm F**, Lowenthal RM, Dickinson JL, Holloway AF, Woods G. Arsenic trioxide and fucoidan synergizes to induce apoptosis in acute promyelocytic leukaemia.
- Poster presentation: 26th International Lorne Cancer Conference (Lorne, Australia 2014). **Atashrazm F**, Lowenthal RM, Dickinson JL, Holloway AF. Induction of Apoptosis by Fucoidan in Human Acute Promyelocytic Leukaemia cells**.**
- Oral presentation: Tasmanian Haematology, Immunology and Neoplastic Group (THING) Meeting. (Port Arthur, Tasmania 2013). **Atashrazm F**, Lowenthal RM, Dickinson JL, Holloway AF. Induction of apoptosis by fucoidan in human leukaemia cells.

Awards resulting from thesis material

2012 International Postgraduate Research Scholarship (IPRS)

2014 David Collins Leukaemia Foundation Professional Development Award for 2014

2014 Travel grant to attend to the 26th International Lorne Cancer Conference

2014 Finalist of the Australian Society for Medical Research (ASMR) Postgraduate Student Competition

2015 Travel grant to attend to the 44th Annual Scientific Meeting, International Society for Experimental Haematology

DEDICATION

This dissertation is lovingly dedicated to my inspirational mother, Azar, for the greatest influence on my life. Her measureless support, help, encouragement and constant love have sustained me throughout my life.

ACKNOWLEDGEMENT

I would never have been able to finish my dissertation without the guidance of my supervisory team, help from friends, and support from my family.

I would like to express my sincerest gratitude to my supervisor, Professor Ray Lowenthal for providing the opportunity to undertake this work with him. I am grateful for his tremendous support and encouragement throughout my research period. I have been extremely lucky to have a supervisor who cared so much about my work and provided an extremely friendly atmosphere on a professional and personal level.

I would like to acknowledge my incredible primary supervisor Associate Professor Jo Dickinson who has supported me throughout my research with her knowledge and outstanding guidance. At many stages in the course of this research project I benefited from her support and I am thankful of her for providing me with an excellent atmosphere for doing research.

A special thank you to my inspirational co-supervisor, Professor Greg Woods. It was an absolute honour and pleasure for me having him as my supervisor in the 3rd year of my PhD. I am grateful for his invaluable helps, and his encouragement and insightful criticism.

I am also grateful to Dr Adele Holloway for her guidance and practical advice during the research project. I am honoured to have her as my co-supervisor who guided me through my PhD study.

I would also like to acknowledge the support I received from staff and fellow students at the cancer genetics group, Menzies Institute for Medical Research (UTAS) especially our research assistant Mr James Marthick for all his technical helps, who always gave full attention to me to solve my problems. I would like to acknowledge Dr Russell Thompson for answering all my statistical questions and Dr Rebekah McWhirter who patiently assisted me for ethics application process.

I am deeply thankful to staff of the animal facility, Menzies Institute for Medical Research (UTAS) particularly Ms Peta Lawrie for teaching me the *in-vivo* techniques and especially for her outstanding patience, enthusiasm, helps and encouragements.

My sincere thanks are extended also to all staff and post-graduate students in Menzies Institute who helped me throughout my PhD. Thank you to Mr Mark Cozens and Dr Terry Pinfold, who assisted me in flow cytometry settings and to Ms Jocelyn Darby for her technical assistance.

I am truly thankful for the tremendous support and love from my friends here in Hobart and in Iran who made this journey incredible, especially Dr Hussain Khan who was always supporting me and encouraging me with his best wishes. I am most indebted for his extreme patience and advice during challenging time of this journey.

Last but not least, special gratitude is conveyed to my lovely family; especially my wonderful parents. Thank you for always loving me, believing in me and supporting me during all the challenges and ups and downs. I am also thankful to my sister, Fatemeh, and my brother, Mehran, for the great joys and happiness they have brought to me.

List of Abbreviations

µg	Microgram
µL	Microliters
µm	Micrometer
µM	Micromolar
ADCC	Antibody-dependent cellular cytotoxicity
ALL	Acute lymphoblastic leukaemia
AML	Acute myelogenous leukaemia
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor 1
APL	Acute promyelocytic leukaemia
ATLL	Adult T cell leukaemia-lymphoma
ATO	Arsenic trioxide
ATRA	All-trans retinoic acid
Bax	Bcl-2 associated X protein
Bcl	B-cell lymphoma
BID	BH3 interacting-domain death agonist
BM	Bone marrow
BSA	Bovine serum albumin
b.w.	Body weight
Ca^{2+}	Calcium
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CLL	Chronic lymphocytic leukaemia
CML	Chronic myelocytic leukaemia
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocytes
CTV	Cell trace violet
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR-4	C-X-C chemokine receptor type 4
Da	Dalton
dATP	Deoxyadenosine triphosphate
DC	Dendritic cell
DD	Death domain
del	Deletion
DIC	Disseminated intravascular coagulation
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DS	Differentiation syndrome
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FAB	French American British

FACS	Fluorescence activated cell Sorter (Flow cytometry)
FADD	Fas-Associated protein with death domain
FCS	Foetal calf serum
FDA	Food and Drug Administration
g	gram
G0	Gap 0
G1	Gap 1
G2	Gap 2
GAPDH	Glyceraldehyde phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GSH	glutathione
h	hour
HCL	Hydrochloric acid
HM	Haematological malignancy
HMW	High molecular weight
HPC	Haematopoietic progenitor cell
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
HTLV	Human T-lymphotropic virus
IAP	Inhibitors of apoptosis protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneal
ITGA	Integrin alpha
IV	Intravenous
kDa	Kilo Dalton
kg	Kilogram
Lin	Lineage
LMW	Low molecular weight
m	Meter
M	Mitosis
M	Molar
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
MEK	MAPK/ERK kinase
mg	Milligram
$Mgcl_2$	Magnesium chloride
miRNA	MicroRNA
mRNA	Messenger ribonucleic acid
mm	Millimetre
MM	Multiple myeloma
MMP-9	Matrix metalloproteinase 9
MNC	Mononuclear cell
MW	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NHL	Non-Hodgkin’s lymphoma
NK	Natural killer
NO	Nitric oxide
NOD-SCID	Non-obese diabetic/severe combined immunodeficiency
OD	Optical density
PAC-1	Procaspase-activating compound-1
PARP-1	Poly (ADP-ribose) polymerase-1
PC	Progenitor cells
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
PB	Peripheral blood
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
PI3K	Phosphatidyl inositol-3 kinase
PI3P	Phosphatidylinositol 3-phosphate
PMA	Phorbol myristate acetate
PML	Promyelocytic leukaemia
PSGL-1	P-selectin glycoprotein ligand-1
RA	Retinoic acid
RARα	Retinoic acid receptor-alpha
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
S	Synthesis
SC	Subcutaneous
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SP1	Specificity protein 1
t	Translocation
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor
TNT	Tris-sodium-tween solution
TRADD	TNF receptor type 1-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
Tris	Tris(hydroxymethyl)amniomethane
uPAR	Uri-kinase receptor
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
V	volt
VEGF	Vascular endothelia growth factor
WBC	White blood cell
WHO	World Health Organisatiom
XIAP	X-linked inhibitor of apoptosis

Table of Contents

Abstract

Leukaemia is the most common form of haematological malignancy and includes a group of diseases characterised by an uncontrolled growth of malignant haematopoietic cells. It accounts for about 33% and 3% of cancer cases in children and adults, respectively. Worldwide, approximately 350,000 new cases of leukaemia are diagnosed each year, with more than 250,000 deaths. Acute myeloid leukaemia (AML) is the most common form of acute leukaemia, accounting for around 85% of adult cases, and a leading cause of cancer death in young adults. Although chemotherapy is often effective in the treatment of AML, at least in the short term, the agents currently in use are associated with a wide range of side effects including increasing the risk of developing therapy-related cancers. Therefore, there is considerable interest in the anti-cancer potential of natural agents with lower toxicities. Fucoidan is a fucose-rich sulphated polysaccharide that exists in the cell wall matrix of brown seaweeds. This component has shown immunomodulatory and anti-tumour activities. However, the underlying mechanisms of these activities remain largely unknown.

Herein, the anti-tumour activities of fucoidan were examined in *in vitro* and *in vivo* models of AML, and its activity as a potential adjunct therapy was investigated. Investigation of the effects of fucoidan on leukaemic cells (NB4; a t(15;17) positive acute promyelocytic leukaemia, KG-1a; a minimally differentiated AML, HL60; a t(15;17) negative acute promyelocytic leukaemia and K562; an acute erythroleukaemia cell line) revealed evidence that fucoidan has a selective inhibitory effect on acute promyelocytic leukaemia (APL) and not other types of AML cells, initiating apoptosis. Examination of the pathways mediating the observed apoptotic mechanisms revealed the observed effect was caspase-dependent as it was significantly attenuated by pre-treatment with a pan-caspase inhibitor. P21/WAF1/CIP1 was significantly up-regulated leading to cell cycle arrest. Fucoidan decreased the activation of ERK1/2, and down-regulated the activation of AKT through hypo-phosphorylation of Thr(308) residue but not Ser(473).

The anti-tumour activity of fucoidan was supported by *in-vivo* evidence which demonstrated that oral doses of fucoidan significantly delayed tumour growth in the APL xenograft model in athymic Balb/c nude mice, potentially by increasing the cytolytic activity of NK cells. This is the first study to reveal the anti-tumour activity of fucoidan on leukaemia *in vivo*. The selective inhibitory effect of fucoidan on APL cells and its protective effect against APL development in mice may prove that fucoidan may be useful in treatment of certain types of leukaemias.

APL is one of the most aggressive types of AML characterised by differentiation arrest and accumulation of abnormal promyelocytes. Current APL therapies are associated with various side effects such as hyper-leucocytosis and differentiation syndrome which occurs in a quarter of the patients and is a serious and potentially fatal complication. There is therefore interest in

the possibility of reducing the incidence of these morbidities through the use of adjunctive therapies which permit lower doses of toxic therapies to be used whilst maintaining efficacy. Herein, the synergistic effects of fucoidan on current APL therapy, arsenic trioxide (ATO) and all-trans retinoic acid (ATRA), were investigated. *In vitro*, the effect of fucoidan combined with both therapeutic and lower doses of these drugs was examined in APL cells. Fucoidan in combination with ATO enhanced apoptosis in APL cells at both therapeutic and lower doses of ATO as indicated by an increased sub G0/G1 population, DNA fragmentation and annexin V positive apoptotic cells. Furthermore, the combination of fucoidan with low doses of ATRA and ATO significantly enhanced cell differentiation as indicated by G0/G1 arrest and increased CD11b expression. The triple combination of these agents resulted in the greatest myeloid differentiation in APL cells compared to single or double combinations.

The efficacy of fucoidan as an adjuvant to the anti-leukaemic activity of ATO or ATRA was identified *in vivo* in athymic Balb/c nu/nu mice bearing APL. Tumour growth was monitored by measurement of tumour size and survival. When fucoidan and sub-therapeutic doses of ATO were administered in APL-bearing mice, the median survival and tumour volume doubling time significantly increased in mice treated with fucoidan alone and combined with ATO but not ATO alone compared to the control group.

When fucoidan plus low dose ATRA were administered as the therapy regimen in APL-bearing mice, the median survival and tumour volume doubling time significantly increased in all treated groups compared to the control group. Moreover, the differentiation of APL cells obtained from animal's tumour mass significantly increased in mice treated with fucoidan alone and fucoidan+ATRA but not ATRA alone compared to the control group. A further novel finding was that the differentiated APL cells derived from the excised tumour mass exhibited a down regulation of CD44 in fucoidan+ATRA treated mice. The presence of differentiated leukaemic cells with low or no expression of CD44 may be associated with decreased migration of these cells in APL.

Taken together these findings provide important evidence that fucoidan may prove an effective adjuvant therapeutic agent in the treatment of selected leukaemia sub-type APL and may permit lower doses of ATO and ATRA to be employed to achieve better efficacy of treatment accompanied by lower toxicity.

CHAPTER ONE

Literature Review

1.1 Haematological Malignancies

Haematological malignancies (HMs) are tumours of the haematopoietic or lymphoid tissues, accounting for nearly 9% of all cancers (excluding non-melanoma skin cancers). Although there is no identifiable cause for most patients, factors such as exposure to ionising radiation, certain chemicals, genetic predisposition, viral infection and Down's syndrome are associated with increased risk of these diseases (1).

Haematological malignancies are classified into three major types; 1. leukaemia; cancer of immature or abnormal white blood cells, 2. lymphoma; cancer of the lymphoid tissues leading to accumulation of abnormal lymphocytes in lymph nodes and other tissues; and 3. multiple myeloma which is cancer of plasma cells originating in bone marrow. Figure 1.1 represents the amount of estimated new cases of leukaemia, lymphoma and myeloma in 2015 in Australia (2).

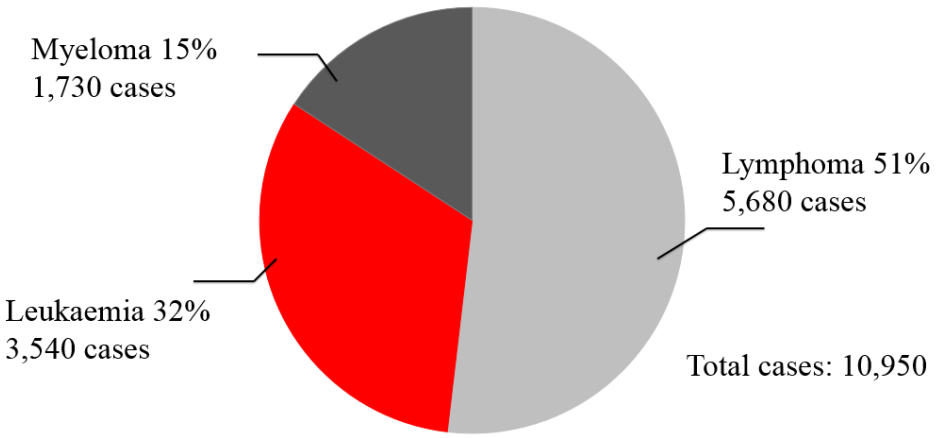


Figure 1.1. Estimated new cases (%) of leukaemia, lymphoma, and myeloma in Australia, 2015*. Source: Australian Institute of Health and Welfare; 2014 (2).
* Total percentage does not add up to 100% due to rounding in the calculation of individual percentages.

1.1.1 Leukaemia

Leukaemias are a group of malignancies involving the uncontrolled proliferation of immature or abnormal white blood cells in bone marrow and peripheral blood (3). Leukaemias are the leading cause of cancer deaths in males younger than 40 and females younger than 20 years

(4). Worldwide, approximately 350,000 new cases of leukaemia are diagnosed each year. There are more than 250,000 deaths. According to a report by Australian Institute of Health and Welfare, 2774 people were diagnosed with different types of leukaemias in 2011, and there were 1368 deaths due to this cancer in 2012 (5).

Haematopoietic cells contain two major lineages; myeloid which gives rise to granulocytes, monocytes/macrophages, megakaryocytes and erythrocytes; and lymphoid which produces different types of lymphocytes (Figure 1.2). Leukaemia may derive from either of these two lineages (6). Leukaemias are also categorised into acute and chronic types depending on the rate of the progression and whether mature or immature WBCs are involved. In acute leukaemias, the number of non-functional immature WBCs increases rapidly without differentiating into mature cells. Acute leukaemias develop quickly and have a poor prognosis if untreated. In contrast, chronic leukaemias mostly involve proliferation of mature WBCs and tends to progress slowly (6).

There are four major types of leukaemias: acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), chronic lymphocytic leukaemia (CLL) and chronic myeloid leukaemia (CML). Among all types of leukaemia, AML causes the most deaths in Australia and worldwide (1, 7).

1.1.2 Acute Myeloid Leukaemia

Acute myeloid leukaemia (AML) is characterised by rapid production and accumulation of immature myeloid cells. It is the most common form of acute leukaemia accounting for 25% of all adult leukaemias in USA, Australia and Europe (8, 9). AML is regarded as a highly malignant neoplasm and has the lowest survival rate of all leukaemias (8). The age of onset of AML has two peaks (Figure 1.3). It is the most common form of acute leukaemia during the first few months of life and also becomes the most frequent form of acute leukaemia in the middle and later years of life, with median age of 60. The increase in incidence of AML could be secondary due to the progression of the very common myelodysplastic syndrome (MDS) in elderly patients to AML. More than 80% of patients with MDS are over the age of 60 (10), and it has been reported that 30% of MDS cases evolve into AML (11).

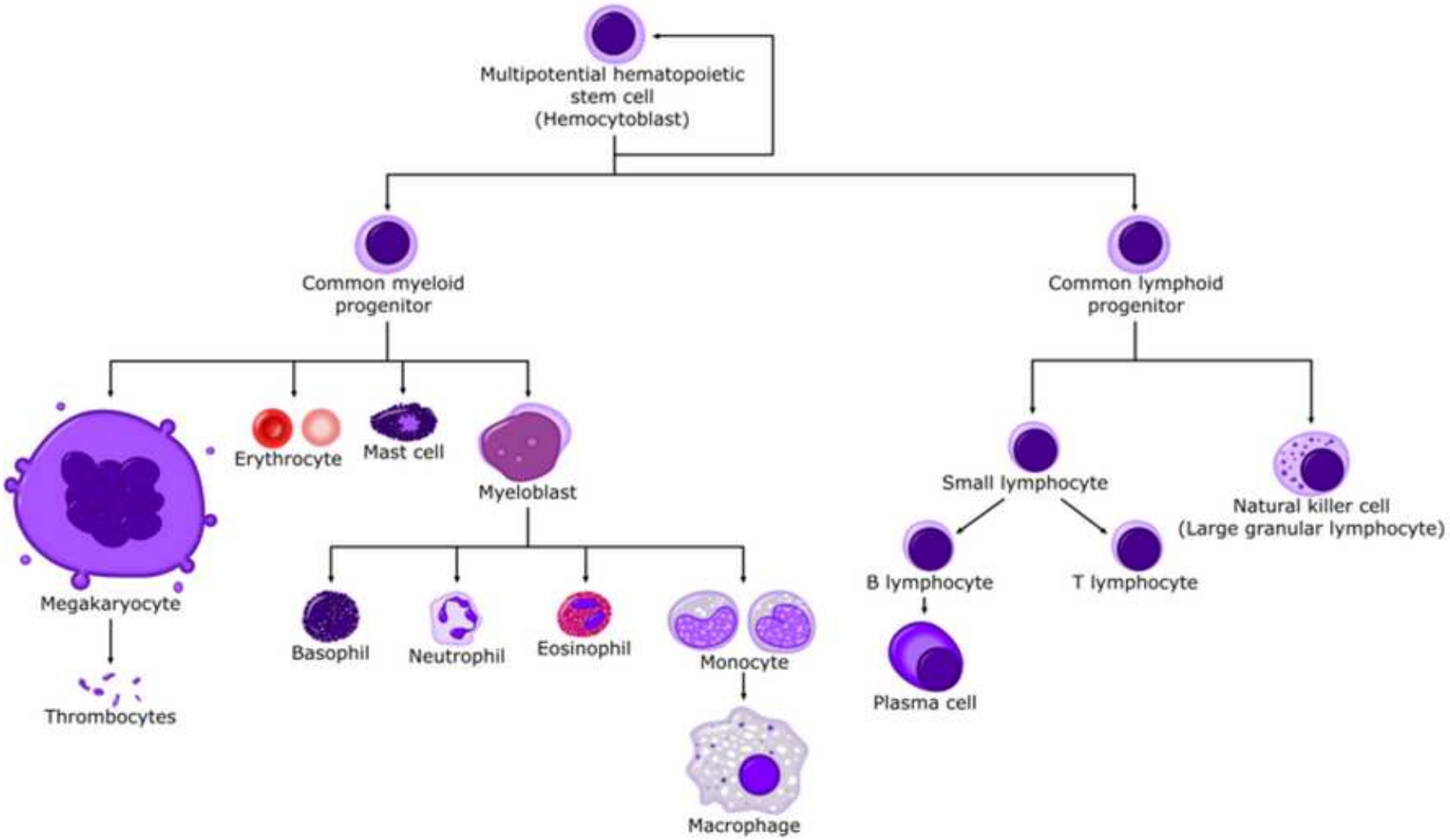


Figure 1.2. Overview of normal haematopoiesis (12)

Various etiological factors have been associated with AML occurrence, such as exposure to radiation, viruses, chemical agents such as benzene and previous treatment with chemotherapeutic agents (6). However, most of the AML cases are not known to be linked with these factors.

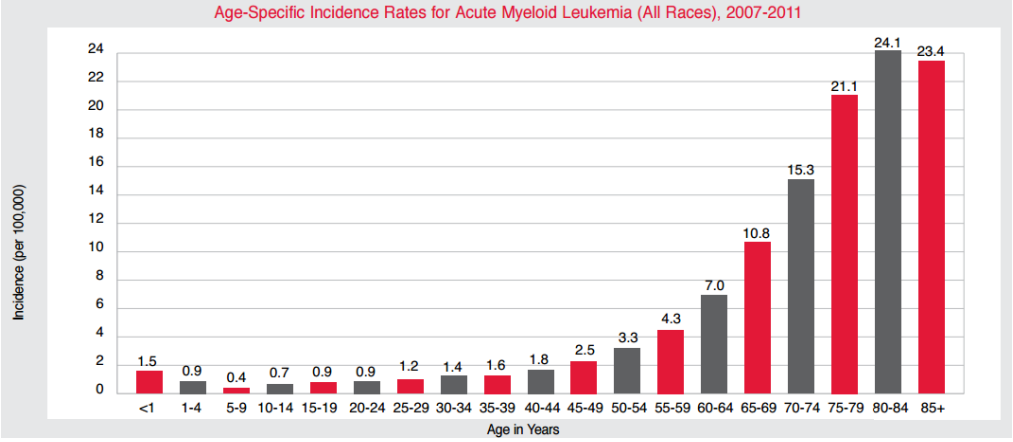


Figure 1.3. Age-specific incidence rates for AML (all races), 2007-2011 (13).

Different subtypes of AML have been categorised. The French-American-British (FAB) classification and the newer World Health Organisation (WHO) classification are two of the main systems that have been used to classify AML. In 1976, the FAB group classified AMLs into 8 different subtypes according to morphology (maturity of the leukaemic cells) and cytochemical reactions of the cells' enzymes (Table 1.1). In 2001, a new classification of AML was introduced by WHO, in which the genetic changes, and biologic and clinical features of the disease were incorporated into the FAB scheme (3). The 2008 revision of the WHO classification of AML is outlined in Table 1.2.

The treatment of AML varies and depends on age, AML subtype and clinical history of patients. The main treatment for AML includes intensive chemotherapy which is composed of two stages: remission induction therapy to eradicate the leukaemic cells and induce remission; and consolidation therapy which begins after remission induction therapy (14). The goal of the latter stage is to destroy any remaining leukaemic cell and to prevent relapse. Other treatment strategies include radiation therapy and stem cell transplantation.

Table 1.1. The FAB classification of acute myeloid leukaemia (6)

Subtypes	Name	Cytogenetics
AML-M0	Undifferentiated acute myeloblastic leukaemia	
AML-M1	Acute myeloblastic leukaemia with minimal maturation	
AML-M2	Acute myeloblastic leukaemia with maturation	t(8;21)(q22;q22), t(6;9)
AML-M3	Acute promyelocytic leukaemia (APL)	t(15;17)
AML-M4	Acute myelomonocytic leukaemia	Inv(16)(p13q22), del(16q)
AML-M5	Acute monocytic leukaemia	Del (11q), t(9;11), t(11;19)
AML-M6	Acute erythroid leukaemia	
AML-M7	Acute megakaryoblastic leukaemia	t(1;22)

Table 1.2. 2008 WHO classification of acute myeloid leukaemia (3)

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1; AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22) CBFB-MYH11 APL with t(15;17)(q22;q12); PML-RARA AML with t(9;11)(p22;q23); MLLT3-MLL AML with t(6;9)(p23;q34); DEK-NUP214 AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1 AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1 *Provisional entity: AML with mutated NPM1* *Provisional entity: AML with mutated CEBPA*
Acute myeloid leukaemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukaemia, not otherwise specified
AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukaemia Acute monoblastic/monocytic leukaemia Acute erythroid leukaemia Pure erythroid leukaemia Erythroleukaemia, erythroid/myeloid Acute megakaryoblastic leukaemia Acute basophilic leukaemia Acute panmyelosis with myelofibrosis
Myeloid Sarcoma

1.1.3 Acute Promyelocytic Leukaemia

Acute promyelocytic leukaemia (APL) or AML-M3 is one of the most aggressive subtypes of AML, characterised by accumulation of abnormal promyelocytes in bone marrow. It accounts for around 10-12% of all AML cases (15) with the unusual average age of 30-40 years, which is remarkably lower than that of other subtypes (60-70 year) (16).

Due to its unique biologic characteristics compared to the other subtypes of AML, APL is characterised by distinct clinical features, associated with an apparent bleeding tendency (6). In the late 1950s, APL was first described as a hyper-acute fatal illness associated with a haemorrhagic syndrome based on this distinctive clinical symptom (17, 18).

Morphologically, abundant amount of azurophilic granules are present within the malignant cells. The clumps of these granules form structures known as Auer rods which are found in most cases of APL (Figure 1.4). The frequently-observed haemorrhagic complications such as disseminated intravascular coagulation (DIC) occur due to presence of pro-coagulant materials in APL cell granules (6).

1.1.3.1 APL Pathophysiology

The chromosomal translocation t(15;17)(q22;q21) is the characteristic chromosomal abnormality in more than 95% of APL cases (19). The t(15;17) translocation involves fusion of segment of the *PML* gene on the long arm of chromosome 15 with segment of the retinoic acid receptor alpha (*RARα*) gene on the long arm of chromosome 17 (Figure 1.5) (20). The *PML* gene encodes the promyelocytic leukaemia protein PML which functions as a nuclear transcription factor and tumour suppressor. The *RARα* gene encodes transcription factor retinoic acid receptor alpha which is mainly expressed in haematopoietic cells. The RARα protein regulates the expression of a wide range of genes involved in proliferation, differentiation and maturation of WBCs, in particular from the promyelocyte stage (21).

The fusion of *PML* and *RARα* genes encodes a hybrid protein, PML-RARα, with altered functions. It attaches to the genes involved in maturation with high affinity, resulting in transcriptional repression of those genes. The affected promyelocytes lose their differentiation capability and cannot give rise to mature cells (22) (Figure 1.6).

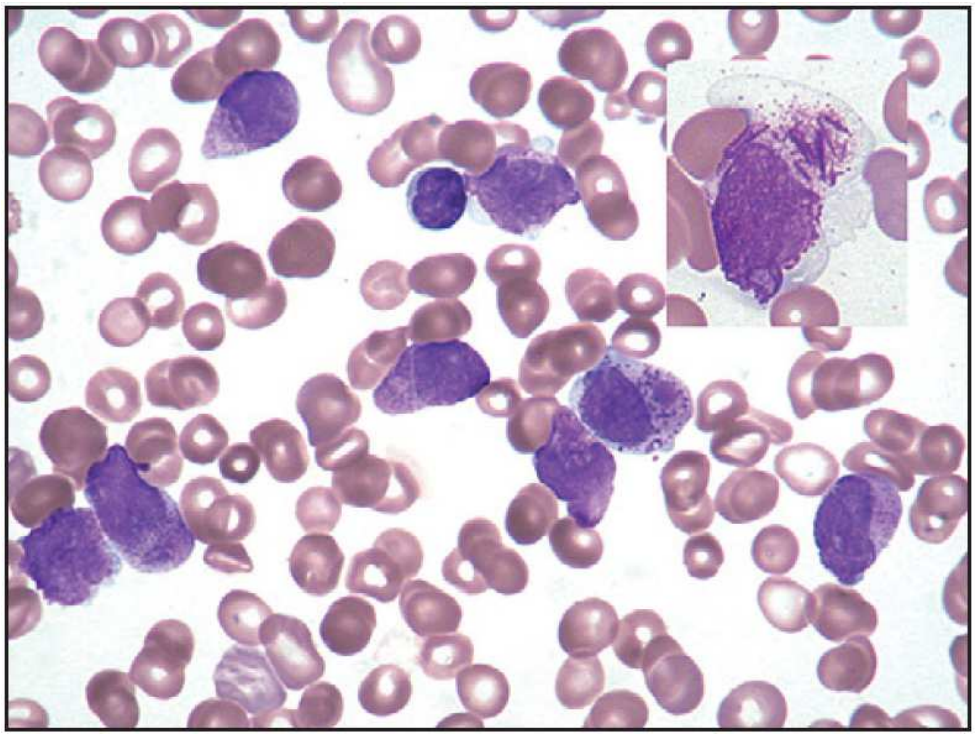


Figure 1.4. Acute promyelocytic leukaemia blood smear. APL with t(15;17) shows predominantly abnormal promyelocytes (×500). *Inset (top right):* Abnormal promyelocytes with bundles of Auer rods. (6)

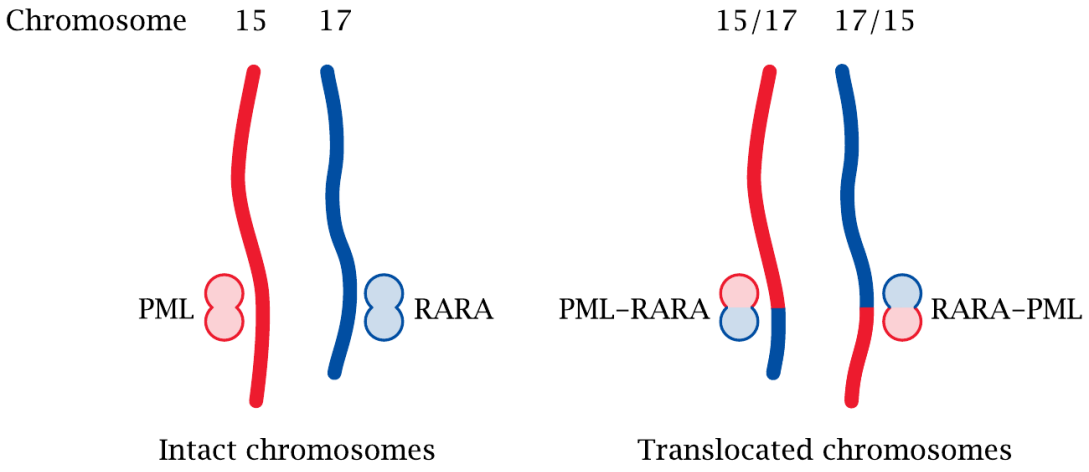


Figure 1.5. Chromosomal translocation between *PML* and *RARα* genes (23)

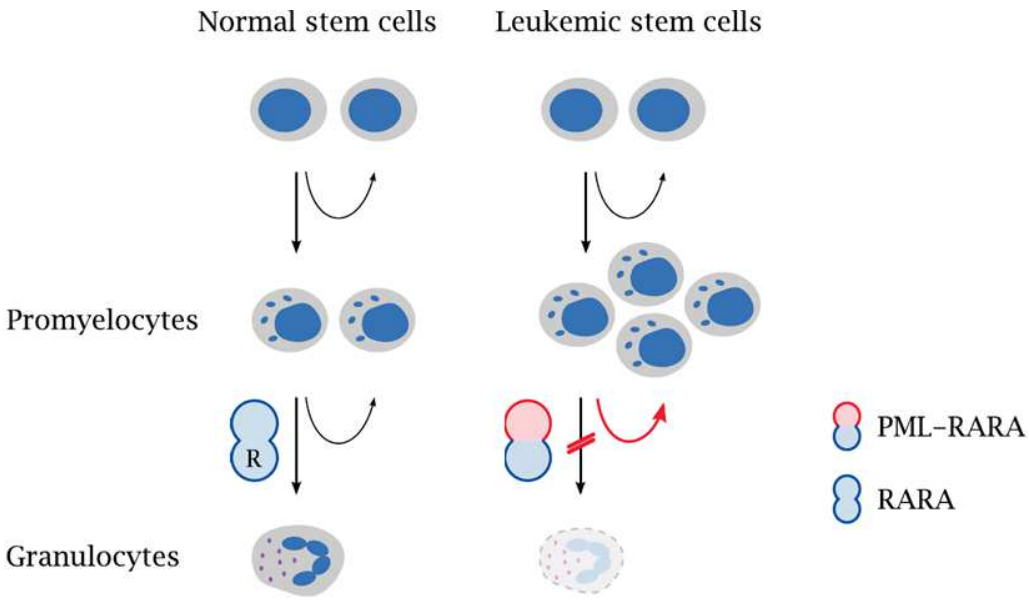


Figure 1.6. The mutant PML-RARα blocks promyelocytes differentiation (23)

1.1.3.2 APL Treatment

At the time of the first description of APL in the late 1950s, the front-line treatment for APL was similar to other subtypes of AML including chemotherapeutic drugs such as daunorubicin. In 1973, due to relatively low rate of complete remission in patients, the remission induction therapy phase was changed to chemotherapy composed of an anthracycline (daunorubicin, idarubicin, or others) and cytosine arabinoside (Ara-C), which led to an increased rate of complete remission from 55% to 75-80% in newly diagnosed patients (24-26). However, due to the unique clinical features of APL and the high likelihood of haemorrhagic complications the death rate was still high and the median duration of complete remission remained low. Since it was discovered that the accumulation of malignant promyelocytes in APL occurs as a result of differentiation arrest, in the early 1970s new insight into developing differentiation-based therapy rather than cell death-based therapy was offered (26). All-trans retinoic acid (ATRA) and arsenic trioxide (ATO) were further introduced as potential molecules targeting differentiation in APL cells. In a newly published clinical trial, the combination of ATO with ATRA and idarubicin was used in induction therapy, and ATO with ATRA were used as consolidation therapy (27).

1.1.3.2.1 All-Trans Retinoic Acid (ATRA)

Retinoids are a family of molecules that are chemically and structurally related to vitamin A (retinol). Retinol, presents in the diet, is stored in the liver and can be converted to various derivatives such as ATRA, 9-cis-RA, 13-cis-RA, and retinal (28). These chemical compounds are present in human plasma and exert diverse physiological functions such as regulation of cell proliferation and differentiation (29).

In 1980s, following introduction of the differentiation-based therapies, the synthetic form of retinoic acid (RA) was shown to trigger morphological and functional maturation in APL cell lines (28). In the first clinical trial, all patients who received ATRA underwent clinical remission, with a gradual differentiation of malignant promyelocytes in bone marrow and peripheral blood. This success was further confirmed by several clinical trials which reported complete remission of 85-90% in patients treated with ATRA (30, 31). However, continuous ATRA therapy led to progressive resistance to the drug leading to relapse within 3-6 months

in almost all patients (32). Subsequently it was found that the combination of ATRA and chemotherapy such as anthracyclines was more beneficial than each alone. This not only resulted in higher overall complete remission rates up to 95%, but also decreased the incidence of differentiation syndrome (DS) in newly diagnosed patients (26). DS is a life-threatening complication which is postulated to be caused by therapy-induced cytokine release from myeloid cells as they undergo differentiation. Clinical features may include fevers, dyspnoea with pulmonary infiltrates, effusions and renal failure (33).

Today, the combination of ATRA and anthracycline-based chemotherapy is the first line of APL treatment in newly-diagnosed patients (22), resulting in 12-year progression-free survival rate of about 70%. However, relapse and death occur in 30% of patients within five years (28).

1.1.3.2.2 Arsenic Trioxide (ATO)

Arsenic trioxide (ATO) or white arsenic is an inorganic form of arsenic derived from minerals such as realgar or orpiment (34). ATO has been used in traditional Chinese medicine for thousands of years. In the late 18^{th} and early 19^{th} centuries, ATO was introduced for treating periodic fever and chronic myelogenous leukaemia. Although its usage was limited as it was highly poisonous and induced severe toxicities in patients (34, 35).

In early 1990s, intravenous injection of crude solutions of ATO containing 1% of the component induced complete remission in 65% of APL patients with 30% survival rate after 10 years (36). Impressively, ATO was shown to be more effective in relapsed APL patients or in patients who developed resistance to chemotherapy or ATRA than newly diagnosed patients (26). In 2000, the Food and Drug Administration (FDA) approved administration of ATO for the treatment of induction of remission and consolidation in patients with APL who are refractory to, or relapsed from, retinoid and anthracycline chemotherapy, and whose APL is characterised by the presence of the t(15;17) translocation or PML/RAR-alpha gene expression (37, 38). Recently, oral formulations of ATO have been developed and are entering clinical practice (39).

1.1.3.2.3 Combination of ATRA and ATO

New insights provided by research using combined treatment of ATO and ATRA led to this chemotherapy being offered in 1998 (26). Subsequently, cell culture and animal studies revealed a significantly increased cell death in acute promyelocytic leukaemia NB4 cell line and a marked increase in survival of APL-bearing animals (40, 41). In the first phase of initial clinical trials it was revealed that even though the complete remission rates in ATRA single, ATO single and combined groups were similar, the time to achieve the complete remission was significantly shorter in the combination group (42). After 18 months follow up, 100% of the cases in the combination group remained in complete remission, while APL relapsed in nearly 20% of the single therapy groups. The combination of oral ATRA and intravenous ATO is now considered as the standard treatment for relapsed APL (39). ATRA plus ATO is also used as induction therapy in elderly patients and in patients who cannot tolerate the anthracycline-based therapy (43). However, despite the promising results from various clinical trials, the efficacy and safety of ATO+ATRA or ATO+ATRA+chemotherapy are still unclear and several pilot studies investigating their use for remission induction in newly diagnosed patients and other stages are ongoing (44, 45). In a recent multi-central randomized clinical trial, Zhu *et al.* tested the efficacy and safety of an oral formula of ATO and compared with intravenous ATO in newly diagnosed APL patients (46). Their findings showed that the oral formula of ATO plus ATRA was not inferior to intravenous ATO plus ATRA as first-line treatment of APL as the disease free survival was 98.1% and 95.5% in oral and intravenous ATO respectively. These results suggest that oral ATO plus ATRA could be considered as first-line treatment option for APL treatment.

Although there have been advances in APL treatment, the early death rate related to hemorrhagic complications is still high. Besides, relapses, drug resistance and significant side-effects of current treatments still remain clinical challenges. Therefore, new APL treatment strategies are needed to increase the disease free survival rate and improve the clinical outcome.

1.2 Cellular Processes in Tumourigenesis

During tumour development, various crucial cellular pathways are disrupted. Over the last decades, the major hallmarks of cancers have been identified as follows; 1. Self-sufficiency in

growth signals, 2. Insensitivity to death signals, 3. Apoptosis evasion, 4. Unlimited replicative potential and immortality, 5. Angiogenesis, 6. Invasion and metastasis to other tissue, (47) 7. Reprogramming energy metabolism and 7. Immune response destruction (48). These acquired capabilities are shared between almost all types of cancers.

Unlike the traditional cancer therapies that kill rapidly proliferating cells and affect both tumour and normal cells, novel therapeutic agents are designed to target the molecules which are abnormally expressed only in tumour cells. These tumour-specific structures could be either surface antigens; such as polymorphic epithelial mucin (PEM) which is aberrantly glycosylated and overexpressed in a variety of epithelial cancers (49), or intracellular molecules; such as phosphatase of regenerating liver-3 (PRL-3) which is overexpressed in gastric carcinoma (50). In this regard, our understanding from the cancer hallmarks and involved pathways has enormously advanced anti-cancer treatments. In this section, the main involved cellular and molecular pathways, the role of their defects in cancer development, and their therapeutic implication in cancer will be reviewed.

1.2.1 Apoptosis

Until recently, accelerated or deregulated cell proliferation was believed to be the only causative factor for cancer. Kerr *et al.* however hypothesised that hyperplasia and malignant neoplasms can result from reduced programmed cell death known as apoptosis rather than increased unlimited proliferation only (51). Apoptosis is an active multi-step cell self-destruct program that can potentially occur in each cell in the body, leading to the removal of unwanted cell.

During apoptosis, the chromosomal DNA is fragmented as a consequence of cleavage between nucleosomes. These DNA fragments can then be broken into small pieces of around 50 nucleotides. Eventually, cleavage of proteins that are responsible for integrity of cytoplasm and organelles leads to cell self-shrinkage and formation of fragments known as aapoptotic bodies (Figure 1.7). The apoptotic bodies can be recognised and phagocytosed by macrophages (52).

Due to the crucial role of apoptosis in abnormal cell removal, the molecules involved in apoptosis are broadly recruited for anti-cancer drug development. Therefore, understanding the apoptotic pathway and involved molecules as well as the role of relevant impairments causing cancers would help in designing therapeutic agents.

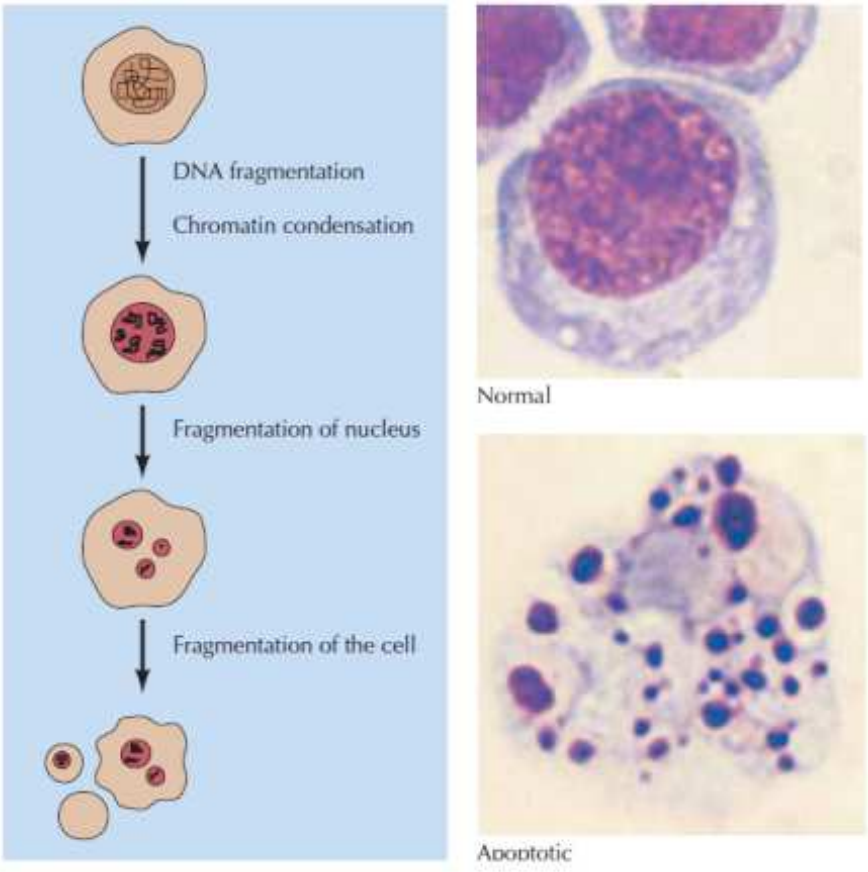


Figure 1.7 Apoptotic bodies (53)

1.2.1.1 Apoptosis Initiation

A variety of exogenous and endogenous agents initiate the apoptosis process including the agents that cause DNA damage (e.g. ionising radiation and cytotoxic drugs such as alkylating agents), the factors that activates the death receptors (e.g. some hormones), the biochemical agents which enhance the downstream components of apoptotic system such as phosphates and kinase inhibitors (e.g. perylenequinone antibiotic calphostin C), and the factors which mediate the apoptosis process by direct cell membrane damage (e.g. heat and other stressors) (54).

1.2.1.2 Apoptosis Pathways

Apoptosis initiation by stimuli results in activation of a number of molecules and proteolytic enzymes, causing the progression of the apoptosis cascade (Figure 1.8). Caspases or cysteine-aspartic proteases known as the executioner proteins in apoptosis cascade are a family of cysteine proteases and have a pivotal role in this process (55). Caspases are divided into initial caspases which trigger the initial steps of the apoptosis; and effector caspases which function at the later stages of apoptosis leading to the cleavage of crucial molecules. The initial phase of apoptosis can be carried out either through activation of the cell death receptors (extrinsic pathway) or via the mitochondrial signalling (intrinsic pathway). In the extrinsic pathway, transmembrane death receptors transmit the death signals to the apoptotic machinery, while the intrinsic pathway is activated in response to death signals derived from inside the cell such as DNA damage. These signals cause permeabilisation of mitochondria and subsequently release of cytochrome C. Both pathways trigger the activation of effector caspases.

1.2.1.2.1 Extrinsic Pathway

A large number of death signals trigger the extrinsic pathway through attachment to death receptors located on cell surface. Different cell-surface death receptors have been characterised so far, which belong to the tumour necrosis factor (TNF) receptor gene superfamily. The Fas receptor, TNF-α receptor-1 and TRAIL receptors DR-4 and DR-5 are

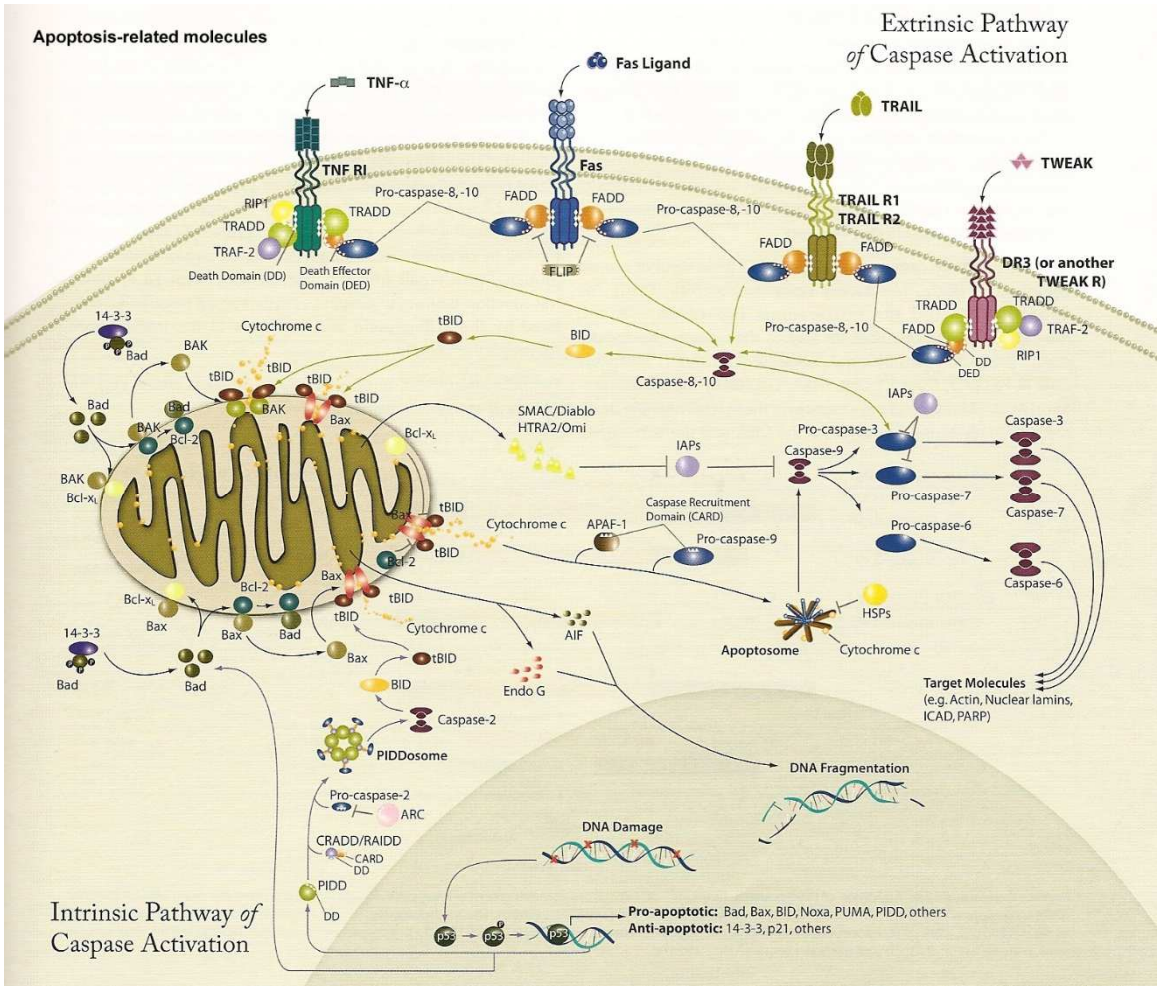


Figure 1.8. Apoptosis related molecules (56)

the main death receptors (Table 1.3). UV radiation is an example of a stimulus which induces the extrinsic pathway of apoptosis through Fas clustering on the surface of keratinocytes.

All death receptors contain a homologous cytoplasmic domain named Death Domain (DD) which includes approximately 80 amino acids and is responsible for transmitting the death signal into the intracellular signalling pathways. The DDs of activated death receptors recruit the DDs in adapter molecules such as FADD and TRADD and cause formation of death inducing signalling complex (DISC) (Figure 1.9). The DISC contains a multi-protein complex such as several caspase 8 molecules. The activated caspase 8 then directly cleaves and activates the downstream effector caspases.

Table 1.3 Death receptors and their ligands

Death receptors	Ligands for death receptors
Fas (CD95)	CD95L (FasL)
TNFR1 (p55, CD120a)	TNF and Lymphotoxin alpha
DR3 (Apo3)	Apo3L
DR4	TRAIL (Apo2L)
DR5 (Apo2)	TRAIL (Apo2L)

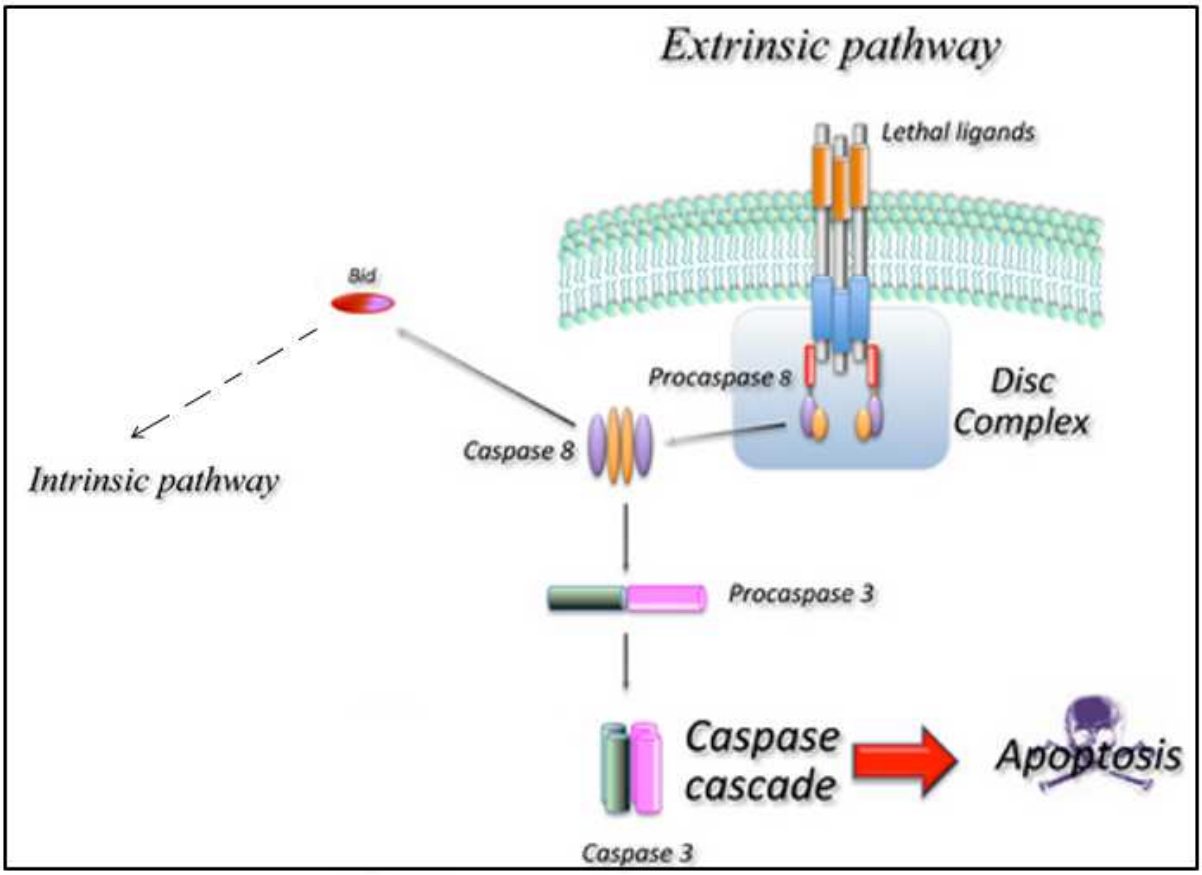


Figure 1.9. Apoptosis extrinsic pathway (57)

1.2.1.2.2 Intrinsic Pathway

Intracellular stimuli and non-receptor mediated signals such as DNA damage, viral infection, hypoxia and high level of Ca^{2+} are responsible for the intrinsic apoptosis pathway. These stimuli induce changes in the inner membrane of mitochondria and lead to mitochondria transmembrane potential loss. Upon this, the mitochondrial membrane permeability increases and pro-apoptotic molecules such as cytochrome C are released into the cytosol. The released cytochrome C accompanied by dATP bind to a protein named Apoptotic Protease Activating Factor 1 (Apaf-1) which is the core of caspase-activating signal complex (Figure 1.10). In the absence of cytosolic cytochrome C, Apaf-1 is in monomeric form, but upon presence and binding to the cytochrome C, Apaf-1 forms an oligomeric holoenzyme complex called apoptosome which triggers the cleavage of procaspase 9 to caspase 9. The activated caspase 9 then activates the downstream executive caspases and promotes the caspase cascade in the later stage of the apoptosis.

Intrinsic and extrinsic pathways can be cross-linked together through a BH3 only family protein named BH3 interacting-domain death agonist (BID). Following the cleavage and activation of BID by caspase 8, the truncated BID is translocated to mitochondria and triggers release of cytochrome C (Figure 1.10).

1.2.1.2.3 Common Pathway

Activation of either caspase 8 through the extrinsic pathway or caspase 9 through the intrinsic pathway results in the activation of effector or executive caspases including caspases 3, 6 and 7. It is believed that caspase 3 is the main executive caspase. In nuclei, caspase 3 cleaves and activates endonucleases such as Caspase-Activated DNase (CAD) which causes DNA fragmentation, degradation and chromatin condensation (52). Another role of caspase 3 is the proteolytic cleavage of poly(ADP-ribose)polymerase-1 (PARP-1), a nuclear enzyme involved in DNA repair and DNA stability. In addition, it mediates the disruption of cytoskeletal molecules such as gelsolin which is the core of actin polymerisation. The cleavage of gelsolin then results in actin filaments depolymerisation and cytoskeletal disruption (58).

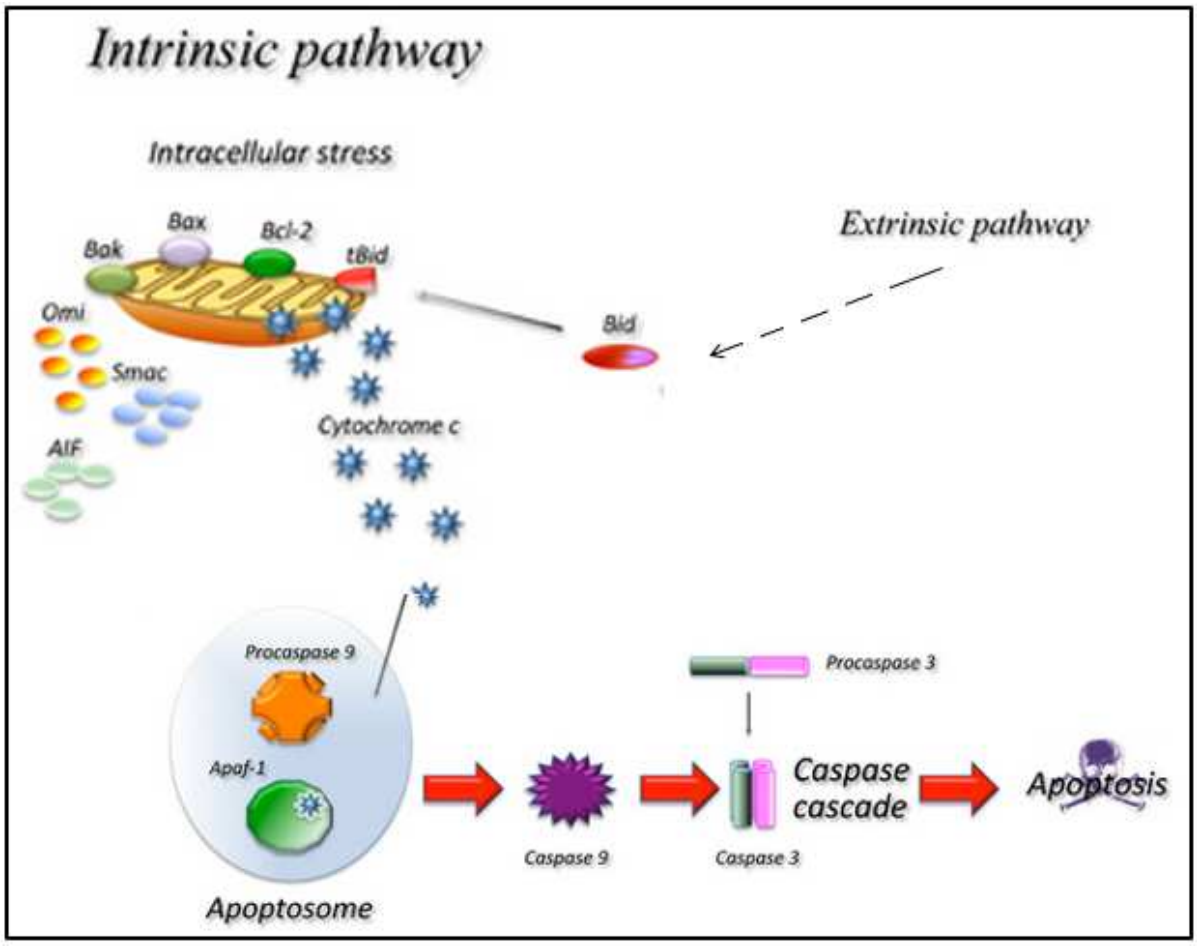


Figure 1.10. Apoptosis intrinsic pathway (57)

1.2.1.3 Apoptosis Regulatory Proteins

Cell survival is regulated by a balance between pro-apoptotic and anti-apoptotic signals. A large group of apoptosis regulatory proteins including oncogenes and tumour suppressor proteins have been characterised (59).

Members of a protein family named Bcl-2 family (B-cell lymphoma 2) play pivotal roles in the regulation of the intrinsic apoptosis pathway. Bcl-2 family includes 25 either pro-apoptotic (such as Bax, Bak, BAD) or anti-apoptotic (such as Bcl-2 and Bcl-xL) proteins that reside in outer membrane of mitochondria and contribute to the regulation of apoptosis pathway. The anti-apoptotic proteins block the release of cytochrome C from mitochondria and inhibit apoptosis cascade permitting cell survival. In contrast, the pro-apoptotic proteins alter the mitochondrial membrane permeability, induce cytochrome C release and promote cell death (60).

Inhibitors of apoptosis proteins (IAPs) are other anti-apoptotic proteins which interact with caspases and inhibit their activation. X-linked inhibitor of apoptosis (XIAP) is one of the IAP members which binds to and inhibits three caspases 3, 7 and 9 (61).

1.2.1.4 Apoptosis Deregulation and Leukaemogenesis

A well-known mechanism for tumourigenesis is disrupted balance between pro and anti-apoptotic proteins. Characterisation and cloning of the protein Bcl-2, one of the main anti-apoptotic proteins in the Bcl-2 family, opened new insight into understanding the impact of apoptosis in tumour development as its mutation and overexpression enhanced cell survival. Bcl-2 derives its name from its original discovery in B-cell lymphomas. The chromosomal translocation t(14;18) involving *Bcl-2* gene on chromosome 18 and immunoglobulin heavy-chain locus (IgH) on chromosome 14 is associated with human B-cell follicular lymphoma. The juxtaposition of these genes results in enhanced transcription and overexpression of the Bcl-2 protein in the cells (62), thereby promoting long-lived follicular precursor and memory B-cells (63).

There are some pro-apoptotic Bcl-2 family proteins whose defect can also cause HM. Many frame-shift mutations in the Bax gene have been identified in T-cell acute lymphoblastic

leukaemia cell lines (64). These mutations lead to a decreased level of proteins or dysfunctional ones and cause impaired apoptosis within the tumour cells.

Another mechanism by which apoptosis contributes to cancer formation is reduced caspase level or function. In 2002, Devarajan *et al*. reported undetectable caspase-3 mRNA levels in breast and cervical cancers and substantially decreased levels of caspase 3 mRNA in ovarian tumour cells (65). The irregular caspase expression has also been shown in HM pathogenesis. In 2003, Takeuchi *et al.* reported frame-shift mutations within the coding repeats of caspase 5 gene in lymphoid leukaemia/lymphoma cell lines (66).

The third mechanism for contribution of apoptosis in tumourigenesis is abnormal death receptor signalling. Several impairments related to death signalling have been clarified so far. Loss of expression of death receptors, non-functional receptors and lack of proper death signals are examples of death receptor signalling defects (67). Fas receptor also known as CD95 or APO1, is a death receptor on the membrane of target cell. Fas ligand or CD95L is a soluble protein which binds to the Fas receptor and causes trimerisation of the Fas receptor. Alterations in Fas receptor or Fas ligand level or function can cause interaction failure or excessive use of these proteins leading to neoplastic transformation. For instance, to balance peripheral lymphocyte homeostasis, Fas is widely expressed on activated T and B-cells. Disruption of the Fas/Fas L pathway has been reported to be highly associated with lympho-proliferation and increased risk of lymphoid neoplasms. For instance, several somatic mutations in *Fas* gene have been identified in non-Hodgkin lymphoma (68).

1.2.1.5 Apoptosis: Therapeutic Implications

In 1975, Searle *et al.* proposed that the chemotherapeutic agents induce apoptosis in the tumour cell (69). Since then, a large number of drugs have been designed, which either inhibit the anti-apoptotic molecules or enhance the expression and function of pro-apoptotic molecules. Here, the anti-cancer agents are classified into drugs which target 1. extrinsic pathway molecules, 2. intrinsic pathway molecules or 3. caspases.

1.2.1.5.1 Therapies Targeting Extrinsic Pathway Molecules

Activation of death receptors by anti-cancer agents such as death receptor agonists has shown promising results in different malignant cells. MegaFasL as an example is a novel Fas agonist and its usage as an effective regimen in haematological malignancy cells has been shown in the last decade (70, 71). However, despite the potential anti-tumour activity of Fas agonists and also TNF agonists, when they were used *in vivo* in murine models, they induced severe toxicity in hepatocytes and also caused haemorrhagic necrosis (72, 73).

TRAIL (TNF-related apoptosis-inducing ligand) is another TNF family molecule which induces cell death through binding to the death receptors DR4 and DR5. TRAIL agonists including recombinant TRAIL natural ligand or monoclonal antibodies also trigger the apoptosis during cancer therapy. It has been shown that TRAIL agonists act more selectively than the other death receptor agonists and their systemic administration to animal models is associated with less toxicity. Also, some pre-clinical studies have demonstrated that recombinant TRAIL or antibodies against DR4 and DR5 enhance the efficacy of radiotherapy. Administration of TRAIL agonists in combination with chemotherapeutic agents such as doxorubicin has brought about profound apoptosis progression in animal models (74).

1.2.1.5.2 Therapies Targeting Mitochondrial Pathway Molecules

This group includes the agents that perform their activities on the inner membrane of mitochondria. The potential anticancer drugs in this group mostly target the Bcl-2 family. Some of these agents inhibit activity of anti-apoptotic proteins such as Bcl-2 and Bcl-xl or down-regulate their expression. Others increase the activity of pro-apoptotic molecules such as Bax and Bad to promote the mitochondrial apoptosis pathway.

“Oblimersen Sodium” is an antisense oligonucleotide which targets the inhibition of Bcl2 protein. At the molecular level, it binds to and degrade the Bcl-2 mRNA, leading to decreased Bcl-2 protein translation. Pre-clinical and clinical studies have indicated that administration of oblimersen sodium combined with chemotherapeutic agents increases their anti-cancer effects. In a clinical trial, administration of oblimersen sodium to CLL patients who had previously received chemotherapy resulted in reduction of hepatosplenomegaly, lymph node size and lymphocyte count (75).

Another strategy includes designing small molecules which inhibit anti-apoptotic proteins. For instance, ABT-737 and ABT-263 bind to anti-apoptotic protein such as Bcl-2 and Bcl-X(L) with high affinity leading to their inhibition (76). ABT-199 is another small molecule Bcl-2 inhibitor, which has recently received breakthrough designation by FDA for certain types of chronic lymphoma leukaemia (77). Findings from a phase I clinical trial showed an overall response rate of 84% in 56 patients with high risk relapsed or refractory CLL, including 20% complete remission (78).

1.2.1.5.3 Therapies Targeting Caspases

Due to the importance of caspases in the apoptosis pathway, targeting the initial and effector caspases is a potential anti-tumour therapeutic strategy. In this regard, some small molecule caspase activators have been developed to activate the effector caspases directly. Procaspase-Activating Compound-1 (PAC-1) is the first-developed procaspase activating agent which promotes apoptosis through triggering caspase 3 activation (79).

Caspase-based gene therapy is another strategy which recruits caspases for promotion of apoptosis. Caspase 8 expression is frequently silenced by transcription factors such as SP1 and ETS-like which are responsible for caspase 8 promoter hypermethylation and inactivation. 5-aza-2'deoxycytidine (5-AZA) is a specific inhibitor of DNA methylation which demethylates the caspase 8 regulatory sequences, enhances the activity of caspase 8 promoter and therefore the expression of caspase 8 leading to apoptosis progress (80).

1.2.2 Cell Cycle

The cell division process is an ordered series of events within cells which lead to cell replication and production of two daughter cells. The processes from one cell division to the another is named "cell cycle" which is characterised by distinct biochemical steps (81). The whole cell cycle takes different amounts of time depending on the organism. For instance, cell division in bacteria like *Escherichia coli* takes around 20-30 minutes, and unicellular yeast divide every

90-120 minutes (82, 83). One complete cell cycle takes around 24 hours in most mammalian cells (81).

1.2.2.1 Cell Cycle Stages

The cell cycle events are classified into four main phases (Figure 1.11). The step where the division of the cell occurs and two daughter cells are formed is known as mitotic phase (M phase). Since each daughter cell needs to receive the same amount of genetic complement from the mother cell, the parent cell needs to duplicate its DNA prior to cell division. The DNA synthesis process is carried out during a stage named synthesis phase (S phase). Furthermore, there are two gaps during the cell cycle; the first gap is located between the end of M phase and onset of DNA synthesis, known as gap 1 phase (G1 phase) and the second gap is between the end of DNA synthesis and onset of M phase (G2 phase). At G1 phase, the cell grows and new organelles in addition to many proteins and RNAs are synthesised. These molecules are required for cell growth and DNA synthesis prior to division. At G2 phase, the repair systems are activated to correct any DNA damage that may have occurred during S phase. Total G1, S and G2 phases are known as a single phase called interphase (84). The majority of the cell cycle process takes place in interphase. In a proliferating human cell, interphase typically needs 20 hours to be completed while mitosis phase takes only less than an hour (81) .

Before cell entry to the S phase, cells might pause in the G1 phase and enter to a state of quiescency called G0 phase. In G0 phase, the cell is neither dividing nor preparing to divide. At any one time, most of the cells in the mammalians body are in G0 phase. Some cell types such as neuronal cells and heart muscle cells remain in G0 phase during the life span of the whole organism, whereas some specialised cells such as haematopoietic, gut epithelium and skin cells are always proliferating (85).

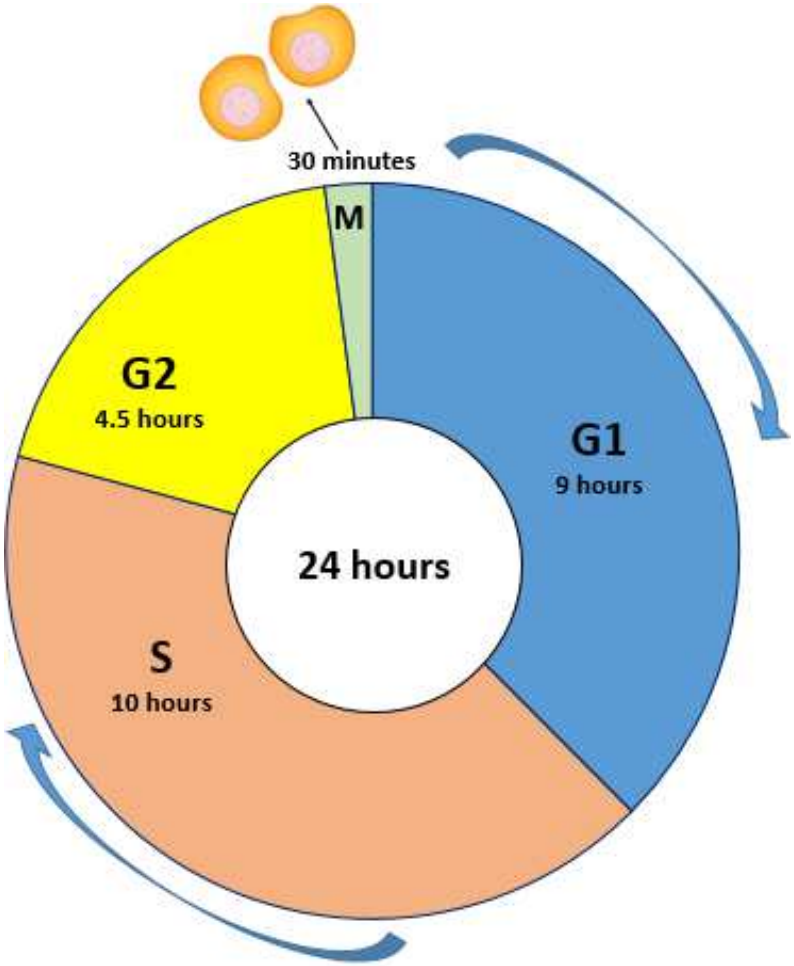


Figure 1.11. Average time spent in each phase of the cell cycle in a human cell

It is important to know that there is a small difference between a G0 cell and a quiescent cell. Quiescent cells are those which have reached a state of terminal differentiation and never re-enter the G1 phase, whereas G0 cells may enter the cell cycle if needed.

1.2.2.2 Cell Cycle Regulation

There are several checkpoints within the cells that prevent them from entering the next step while they have not completed the previous step. Also, there should be certain checkpoints which allow G cells or newly divided cells to re-enter the cell cycle.

The central molecules which positively regulate the cell cycle are called cyclin-dependent kinases (CDKs). CDKs are small serine/threonine protein kinases that have low enzymatic activity. CDKs have regulatory subunits referred as “cyclins” whose attachment to CDKs leads to CDK activation. CDKs 1, 2, 4 and 6 and cyclins A, B, D and E are the main CDKs and cyclins that function in specific phases of the cell cycle (81). CDKs 2, 4 and 6 are G1-specific CDKs. Cyclin D-CDK 4-6 complexes help the cell to progress toward late G1 phase. CDK2 however functions in late G1 phase and is responsible for G1 phase completion and cell entry to S phase (81). Progression from either S to G2 or from G2 to M phase is dependent on cyclin A expression (85) whereas cyclin B-CDK1 complex promotes M phase progression (86).

A different group of proteins have been identified as negative regulators of the cell cycle. These proteins are referred as cyclin-dependent kinase inhibitor protein (CDKIs or CKIs). CDKIs which function as tumour suppressor genes normally inhibit G1-specific CDKs and lead to cell cycle arrest at G1 phase. p16, p19, p21/WAF1 and p27 are examples of CDKIs which inhibit CDK2, CDK4, CDK6 or G1 related cyclin-CDK complexes (81).

Cell cycle progression is also regulated by a variety of external signals such as growth factors and stress stimuli. These stimuli affect cell cycle through different signal transduction pathways. The Ras/Raf/MAPK pathway is one of the main signalling pathways which controls the cell cycle mainly through the activation of G1 cyclin-CDK complexes (87).

1.2.2.3 Cell Cycle Deregulation and Leukaemogenesis

The final aim of the cell cycle is to replicate exactly one copy of DNA, not more and not less, to produce two identical chromosomes during synthesis phase and then to segregate the chromosomes equally between two daughter cells. Defects in the process can lead to serious abnormalities.

The role of deregulation of the cell cycle components in haematological malignancy development and progression has been well established. Due to the high rate of haematopoietic cell turn over, maintaining the balance between cell death and cell proliferation is an essential element for normal haematopoiesis. B-cell chronic lymphocytic leukaemia (B-CLL) is a well-identified example of haematological malignancies in which tumour cells accumulate through reduction of cell death process rather than high proliferation. In contrast, Burkitt lymphoma is a type of haematological malignancies which is characterised by high proliferation index and increased activity of cell cycle promoters.

In 2010, Gladkikh *et al*. examined the expression of cyclin D1 in different types of lymphomas compared with normal mature B-cells and showed a four-fold increase in cyclin D1 expression in mantle cell lymphoma (MCL) compared to normal lymphocytes (88). Cyclin E is also a proto-oncogene whose amplification and overexpression have been reported in ALL and AML (89, 90).

As discussed above, CDK inhibitors negatively regulate cell cycle. The deregulation of CDKI gene has been reported in leukaemia development. p16INK4 for instance is a critical regulator of CDK activity, which specifically binds to CDK4 or CDK6 and inhibits cell cycle progression (91). The homozygous deletion of chromosome 9 (del9p21) containing *p16INK4* gene resulting in its complete or partial loss has been documented in different types of haematological malignancies (92).

1.2.2.4 Cell Cycle: Therapeutic Implications

Over the last several years, our understanding about the role of genetic alterations in CDKs, CDKIs and other cell cycle molecules in carcinogenesis have opened new insights into designing anti-cancer agents which target these molecules. CDKs, which are the key motor of

cell cycle progression are known to be upregulated in malignancies. Many novel synthetic CDK inhibitors have been developed to inhibit CDKs expression and induce cell cycle arrest in the tumour cells. These anti-cancer agents can either directly target the catalytic activity of CDKs or target the regulatory mechanisms that contribute to CDK activity.

Flavopiridol is a pan CDK inhibitor, which binds to, and inhibits CDKs 1, 2, 4, 6 and 7 and leads to cell cycle arrest at the G1 to S as well as G2 to M points. Several clinical trials have assessed the proliferation inhibitory effect of flavopiridol on a variety of human malignancies. In a clinical trial, a novel schedule with specific doses of flavopiridol was employed in patients with refractory CLL who had high-risk cytogenetic abnormalities. It was reported that 45% of patients attained a high response with durable remissions (93).

Seliciclib or CYC202 is another CDK inhibitor, which inhibits multiple CDKs. This agent selectively blocks CDK2–cyclin E, CDK2–cyclin A, CDK7–cyclin H and CDK9–cyclin T1. CYC202 has been shown to increase the sub G0/G1 population and down-regulates cyclins D1 and H in mantle cell lymphoma cell lines (94).

1.2.3 Signal Transduction Pathways

The signals created by extracellular stimuli are transmitted to the downstream effector cells by a series of phosphorylation steps through pathways known as signal transduction pathways. The transduced signal into nuclei up-regulates or down-regulates certain genes on DNA, which are associated with various cellular processes such as proliferation, growth, apoptosis and differentiation (95). Ras/Raf/MEK/MAPK, PI3K/Akt, JAK/STAT and Wnt signalling pathways are examples of signal transduction pathways which play key roles in cell proliferation and apoptosis.

The Ras/Raf/MEK/MAPK pathway is one of the best categorised signal transduction pathways in cell biology (96). The external stimuli such as growth factors first activate Ras protein by GDP-GTP exchange (Figure 1.12). Activated Ras phosphorylates and activates different transduction molecules like serine-threonine kinase Raf. Activated Raf then phosphorylates MEK (MAPK/ERK kinase), which subsequently phosphorylates MAPK (Mitogen-Activated Protein Kinase) molecules such as ERK1 and ERK2. Upon phosphorylation, the activated

ERK1 and ERK2 translocate into nuclei where they activate their substrates; transcription factors which can further change gene expression (97).

PI3K/Akt/mTOR is another important signalling pathway that regulates numerous cellular functions such as angiogenesis, metabolism, survival and apoptosis. The proto-oncogene AKT is the main enzyme in this pathway, which mainly inhibits apoptosis (98). Upon the pathway activation, phosphatidylinositol tri phosphate (PIP3) is produced by phosphoinositide 3-kinase. PIP3 acts as plasma membrane docking site for proteins such as AKT (Figure 1.12). Akt is then phosphorylated and activated by kinase activity of another molecule and transduces the signals into nuclei through phosphorylation and activation of downstream molecules (99).

1.2.3.1 Signal Transduction Pathway Deregulation and Leukaemogenesis

Defects in signal transduction pathways are highly linked with oncogenic transformations. The ERK pathway is the best-studied mammalian cell signalling pathway and its deregulation has been associated with one-third of all human cancers (96). The ERK pathway is mostly associated with cell proliferation, thus its overexpression contributes to tumourigenesis. As explained above, Raf and Ras groups are the initiator molecules of this pathway. The mutations occurring on these early stage molecules are the leading causes of consecutive activation of ERK pathway resulting in cancer development. For instance, B-Raf mutation alone is found in 66% of malignant melanomas and is seen in various other malignancies such as colon and pancreatic cancers at lower frequencies (100). The ERK pathway deregulation has been reported in haematological malignancies as well. Okuda *et al.* showed that hyperphosphorylation and activation of proto-oncogene Raf-1 is highly associated with clonal evolution of myeloid leukaemic cells. Ras mutations are also seen in nearly 40% of patients with MM and in about 30% of myelodysplastic syndromes and AMLs (101, 102).

PI3K/Akt/mTOR contains various oncoproteins and tumour suppressors, and deregulation of these components can mediate tumour development. Constitutive activation of PI3k/AKT pathway has been reported in half of AML cases (103). As AKT is a survival factor, its overactivation is highly associated with tumour survival and drug resistance. Blackburn *et al.*

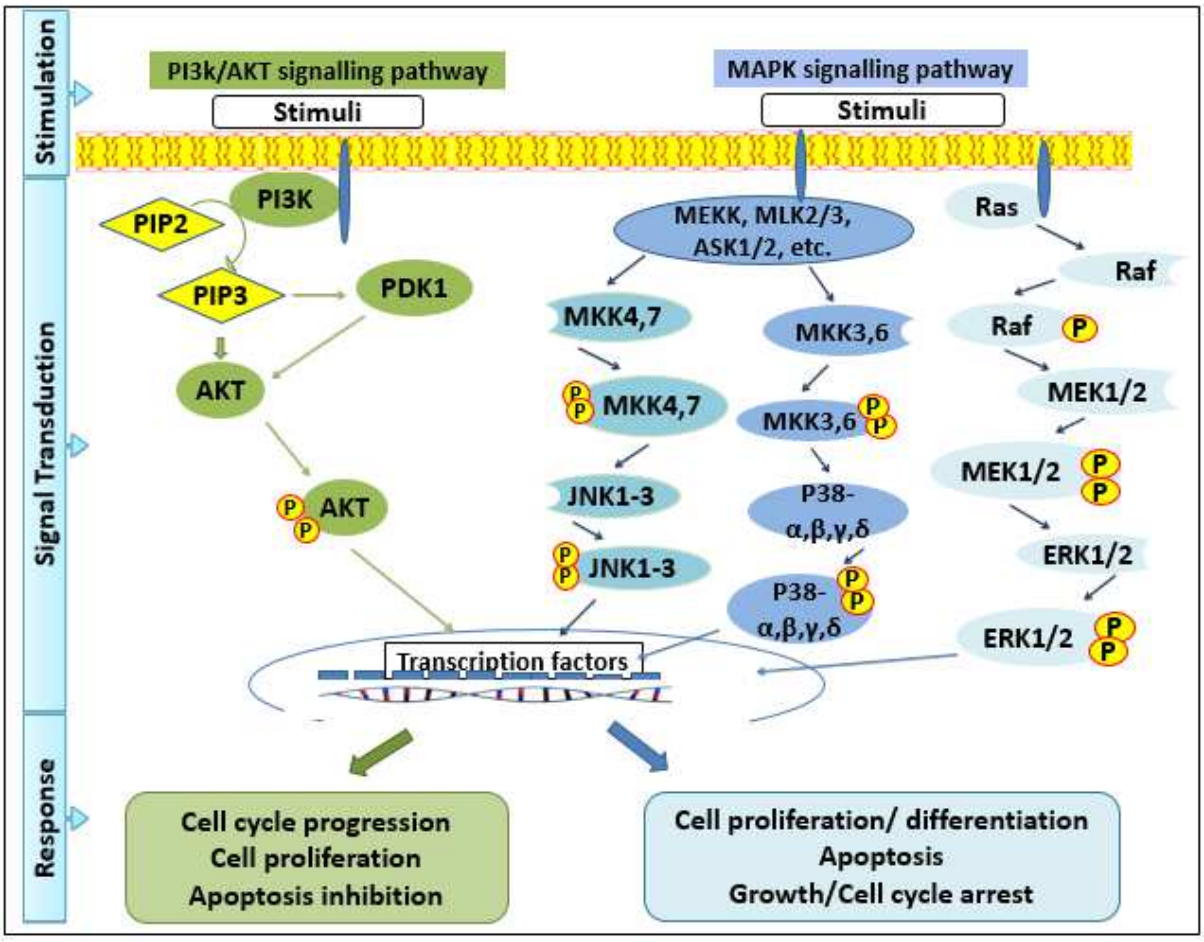


Figure 1.12. Overview of main signal transduction pathways involved in cell proliferation and apoptosis

reported that at least 50% of relapse-driving leukaemic cells evolved genetic lesions and epigenetic modifications that acquired AKT activation (104). They showed that inhibition of AKT pathway in acute lymphoblastic leukaemia restored the malignant cell responses to chemotherapy.

1.2.3.2 Signal Transduction: Therapeutic Implications

Signalling pathway molecules have high potential to be exploited as targets for cancer treatment. As mentioned, mutant up-regulated B-Raf is commonly seen in various cancers and therefore B-Raf inhibitors are amongst the best potential therapeutic candidates. Two B-Raf inhibitors namely BAY43-9006 (Sorafenib) and PLX4032 (Vemurafenib) have obtained FDA approval and are in clinical use in advanced renal cell and hepatocellular carcinomas and in late-stage melanoma, respectively (105).

RAS genes are the most commonly mutated oncogenes in human cancers and thus designing therapeutic agents inhibiting Ras activity is of considerable interest. Enzyme Farnesyl transferase (FTase) is responsible for activating the inactive cytosolic Ras. Synthetic inhibitors of FTase (FTIs) have shown promising activities in treating haematological cancers in clinical trials (106).

Inhibitors of PI3k/AKT/mTOR pathway are other examples of anti-cancer agents. Administration of these agents caused partial response in patients with gynaecological and breast cancer (107). It is also well established that inhibition of PI3k/AKT/mTOR pathway can restore the tumour cells sensitivity to chemotherapy and radiotherapy. Therefore, their combination with chemotherapy can increase the efficacy of standard treatments (108). Amongst the PI3k inhibitors in development, idelalisib is the most studied compound in haematological malignancies, and was recently granted a full FDA approval to treat patients with relapsed CLL (109, 110).

1.2.4 The Role of Immune System in Tumourigenesis

Immuno-oncology research has provided strong evidence that tumour cells can be recognised by the immune system and destroyed or controlled by complex immune pathways. Cancer development has been shown to be accompanied by immune suppression in many types of

cancers, leading tumour cells to escape from immune system. The mechanism of this escape varies. Loss of tumour antigens, which normally make them recognisable to immune cells, and loss of tumour cell sensitivity to T-cells or natural killer (NK) cells are examples. Moreover, alterations in cytokine production by tumour cells or immune cells can provide a beneficial tumour microenvironment for cancer cells, as those can inhibit the immune cell activity (111).

Our understanding from the mechanisms by which immune cells and molecules lose their immunosurveillance ability has opened new insights into developing immunotherapy approaches for cancer treatment. Designing these strategies has helped us to boost immune system responses against cancers. These strategies, for instance, can suppress the unfavourable tumour microenvironment which is caused by suppressive cytokines produced by tumour cells and immune cells. Specific anti-tumour immunity has been achieved by using antibodies designed for certain tumours or using agents which induce T-cell expansion (112).

1.2.4.1 Immune Response: Therapeutic Implications

Over the last two decades cancer immunotherapy (exploiting immune system mechanisms to treat cancers) has become a main focus of anti-neoplastic agent research. One of these approaches is to administer cancer vaccines by which the immune system would be able to recognise tumour cells. Another method is the use of passive immunotherapy by administration of monoclonal antibodies. The designed antibodies are specific for tumour specific antigens and therefore their binding to the tumour cell surface helps the immune system to recognise and kill the tumour cells. This approach promotes antibody-dependent cellular cytotoxicity (ADCC), whereby immune cells such as NK cells recognise the antibody-coated tumour cells, bind to them and destroy the cancer cells by cytolytic activity.

Rituximab, a monoclonal anti-CD20 antibody, is one of the best categorised chimeric monoclonal antibodies, which is widely used in patients with certain types of leukaemias and lymphomas. CD20 is highly expressed on neoplastic B-lymphocytes and using anti-CD20 along with chemotherapeutic agents has improved the outcome of patients with B-cell neoplasms. It has been identified that binding of rituximab and CD20 triggers NK-cells to mediate ADCC (113).

Targeting immune checkpoints that maintain physiologic self-tolerance has been a focus of cancer immunotherapy in recent years. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a protein receptor that regulates the immune system and tolerance through inhibition of T-cells. Clinical and pre-clinical data have shown that blockade of CTLA-4 using anti-CTLA-4 monoclonal antibody therapy is a promising approach for a number of cancers, particularly advanced melanoma (114). It is believed that suppression of inhibitory signals leads to the generation of an antitumor T-cell response, and therefore CTLA-4 blockade results in direct activation of CD4+ and CD8+ effector cells (115). Programmed cell death protein 1 (PD-1) is another immune checkpoint, that down-regulates immune system by preventing the activation of T-cells, and its blockade by monoclonal anti-PD-1 antibodies restores the immune function in the tumour microenvironment in melanoma, renal and non-small cell lung cancers (116).

Another approach of cancer immunotherapy is use of immune cells such as NK cells and cytotoxic T-cells. To increase the cytotoxic activity of immune cells in patients, these cells are isolated from the patient's body, activated *in vitro*, enriched and then transfused back to patient (117). Another method is to administer therapeutic cytokines such as alpha/beta interferons to stimulate and activate the immune cells *in vivo* (118).

1.2.5 Cancer Therapy Complications

Despite promising tumour growth-inhibitory effects in pre-clinical tests, many potential therapies fail in clinical trials when adverse unexpected side effects become apparent. Traditionally anti-cancer chemotherapy targets rapidly dividing cells. Therefore, normal cells which have high-proliferating potential are also affected. Novel therapeutic agents are designed to target specific molecules (targeted therapy). However, these targeted therapies are not always completely free of side effects either. For instance, usage of the B-Raf enzyme inhibitor vemurafenib, the first targeted molecular therapy, is highly associated with a rapid emergence of acquired resistance and adverse dermatological effects. It also stimulates B-Raf expression in V600E B-Raf negative patients, promoting melanoma growth (105, 119). Monoclonal antibodies are another example of targeted therapy as they specifically target the cancer antigens located on tumour cells. Monoclonal antibodies are generally safer than chemotherapy and the side effects mainly include mild allergic reactions such as urticaria. However, they can

also cause severe reactions such as infusion reactions and serum sickness. Rituximab (anti-CD20), as an example, generally causes only mild toxicities. However, severe complications such as anaphylactic reactions, high risk of tumour lysis syndrome in patients who have high burden of circulatory tumour cells, and myocardial infarction have been reported (120).

1.2.6 A Role for Natural Products for Cancer Treatment

Toxicity, drug resistance and increased risk of developing secondary cancers from chemotherapeutic chemicals often limit their efficacy in cancer treatment. Concerns over these complications have generated interest in exploiting natural products, as they are generally safer and have multi-functional mechanism of action for cancer treatment. In the last decades, organic substances derived naturally from microbes and plants; either in their unmodified (naturally occurring) or synthetically modified analogues, have provided a number of useful cancer chemotherapeutic drugs (121). Taxol is an examples of these molecules, which is used to treat a number of cancers such as breast and colon cancers. It was first isolated from bark of the Pacific yew, *Taxus brevifolia* (122).

Due to the multi-functional properties, natural products are also being tested as adjuvants for use in synergy with chemotherapeutic agents. For example those with immunomodulatory effects can reduce immune suppression and the associated increased risk of infection. In George *et al*. (123) study, *Indukantha Ghritha*, a polyherbal preparation consisting of 17 plant components, was used as an adjuvant to cyclophosphamide cancer chemotherapy and shown to stimulate the haematopoietic system and induce leucopoiesis in tumour-bearing mice. When administrated in combination with cyclophosphamide, it reversed myelosuppression induced by cyclophosphamide suggesting its potential to minimise or reverse chemotherapy-induced leucopenia.

Polysaccharides include a large family of diverse biopolymers constituted by monosaccharide residues linked together by *O*-glycosidic linkage and can be seen in both natural and semi-synthetic forms (124). Due to structural diversity, polysaccharides display the highest biological properties among macromolecules. Many natural polysaccharides obtained from natural sources such as plants and algae have demonstrated anti-cancer properties. The multifunctional structure of natural polysaccharides also allows them to be used in conjugation

with anti-cancer agents which lack physiochemical and biopharmaceutical properties (124, 125). Among those, fucoidan derived from brown seaweeds has gained significance in the treatment of cancer.

1.3 Fucoidan

(This section has been substantially published in Atashrazm et al. 2015 (126)*)*

Seaweeds have been a part of diets in China, Japan, and Korea since ancient civilization. Approximately, 400 different types of seaweeds are consumed by humans as food and for medical purposes (127). In the 11th century, in "The Canon of Medicine" -an encyclopaedia of medicine compiled by Persian polymath Avicenna (Bu-Ali Sina)-, the medical values of algae of different species were described. Among those, the usage of marine algae in treatment of tumours, vesicles, and joint pain has been proposed (128).

Polysaccharides are of the main functional components in the structure of seaweeds. Fucoidan is a sulphated polysaccharide that exists in cell wall matrix of different species of brown seaweeds such as mozuku, limumoui, bladderwrack and wakame. Variant forms of fucoidan have been also recognised in some marine invertebrates (129) such as sea urchins (130) and sea cucumbers (131). The first isolation of fucoidan was carried out by Professor Kylin from a slimy ingredient of Kombu in 1913 (132). Research studies based on fucoidan functional activities and biological properties started to gain importance in early 1970's. These studies have reported the potent physiological and biochemical properties of fucoidan such as its cytotoxic activities (133-135).

Due to its heterogeneity and natural source, fucoidan remains unexploited as a basis of therapeutics. It is believed that pharmaceutical companies prefer "druggable" small molecules rather than larger and polydisperse compounds (136). In recent decade, few companies have attempted to produce fucoidan for research use. In limited studies, commercially available heterogeneous fucoidans have been examined in several clinical trials mainly for their anti-coagulant and anti-viral properties. It is suggested that reproducibly defined fucoidan fractions could be created from controlled sources of seaweed, using modern extraction and characterization methods. As suggested by Fitton (136), high quality defined fucoidan could

be produced to standards suitable as ingredients in medical devices and even cross the threshold into drug development route".

1.3.1 Fucoidan Structure

Fucoidan is a heterogeneous sulphated polysaccharide and its chemical composition differs based on the seaweeds source, harvesting time, and local climate conditions (137, 138). Fucoidan mainly consists of sulphated L-fucose with a fucose content of 34-44% (139) and can contain a large quantity of other monosaccharaides such as galactose, mannose, xylose and uronic acid all of which make up less than 10% of the total fucoidan structure. The degree of sulphation varies between 4-8% based on the harvesting location and season (138). The molecular weight (MW) of fucoidan also differs mainly according to the type of the seaweed from which it is isolated (132).

1.3.2 Fucoidan Sources

Fucoidan is widely extracted from different sources of seaweeds such as *Fucus vesiculosus*, *Cladosiphon okamuranus* and *Sargassum muticum*. Among those, *Undaria pinnatifida* and *Fucus vesiculosus* are two major sources of fucoidan that have been shown to have powerful biological activities *in vitro* and *in vivo*.

1.3.2.1 Undaria pinnatifida

Undaria pinnatifida also called wakame or Japanese kelp can reach an overall length of about 0.5-3 metres (Figure 1.13). Wakame is edible seaweed and is widely consumed in Asian countries. The extracted fucoidan from this alga has been classified by the United States FDA as GRAS (Generally Regarded as Safe).

This seaweed is native to Japan, Korea and China, however, it has been spread out from its origin to other sites of the world such as the Atlantic and Mediterranean coasts of Europe (140-142) and shores of New Zealand (143), Argentina (144) and Australia (145, 146).

1.3.2.2 Fucus vesiculosus

Fucus vesiculosus is the scientific name for brown macro algae named bladderwrack. It contains a main midrib while paired spherical vesicles surround it (Figure 1.14). Its colour varies from light yellow to brownish-green and its maximum length reaches to around 2-3 feet. Bladderwrack is found on the coasts of the North Sea and the Atlantic and Pacific Oceans.

Figure 1.13. ***Undaria pinnatifida***

Figure 1.14. ***Fucus vesiculosus***

1.3.3 Fucoidan Metabolism

Fucoidanase, the enzyme responsible for fucoidan hydrolysis, has only been found in brown seaweed and marine microorganisms such as some marine bacteria and fungi (147) and not in humans. It is possible that the acidic conditions in the stomach could degrade fucoidan, however it has been reported that the low gastric pH has restricted effects on fucoidan (148).

Transport across Gut

Investigations over fucoidan transportation across the gut have shown that small amounts of dietary fucoidan can be endocytosed and cross the intestinal wall directly without breaking down (148). In Tokita *et al*. study, 10 volunteers were given oral fucoidan and the concentrations of fucoidan in the serum and urine were analysed. Fucoidan was detectable 3 hours after administration and increased to 100 ng/mL in serum and 1000 ng/mL in urine.

Although, the rate of absorption in the small intestine was highly variable among the participants. The MW of fucoidan in serum was similar to administered fucoidan indicating that fucoidan was not hydrolysed by digestive enzymes (149). However, the MW of the fucoidan detected in urine was significantly smaller than the ingested fucoidan suggesting that fucoidan is degraded in the excretory system and possibly the kidney and not by intestinal enzymes or normal flora.

Transport across Cell Membrane

To evaluate the fucoidan uptake process by cells, the internalisation of low molecular weight (LMW) fucoidan into rabbit smooth muscle cells (SMCs) was analysed. Fucoidan was shown to be internalised by endocytosis at 6 hours. The number of vesicles containing fucoidan increased in the peri-nuclear region at 24 hours, but nuclear internalisation was not observed at any time during the study (150). However, examining the transport of a native fucoidan from *Cladosiphon okamuranus* with MW of 80 KDa revealed a poor permeation of fucoidan across the human colon adenocarcinoma Caco-2 cell monolayer (151).

Regarding the specific ligands by which fucoidan binds to the cell surface, several molecules have been proposed including class A macrophage scavenger receptors for fucoidan attachment to macrophages (152) as well as adhesion molecules such as integrins (153), L-selectin and P-selectin (154). However, Aisa *et al*. reported the anti-tumour activity of fucoidan on human

HS-Sultan lymphoma cells while these cells lack selectin on their surface indicating that fucoidan exerted its activity through selectin-independent mechanisms (155). Two main pathways have been described for receptor-mediated endocytosis including the clathrin-dependent and the caveolae/lipid raft-dependent endocytic pathways. In recent studies, it has been revealed that endocytosis of fucoidan is performed by caveolae-dependent pathway (156).

1.3.4 Fucoidan Biological Properties

A century after discovery of fucoidan, only recently, the biomedical potentials of fucoidans have been elucidated. It has been reported that this agent has effect on the immune system and can enhance rapid haematopoietic stem cell mobilization (134). Kim *et al.* studied the immunomodulatory effects of fucoidan on dendritic cells and indicated that viability of dendritic cells and production of some important immune system proteins such as TNF-α and IL-12 increased in the presence of fucoidan (157). Additionally, fucoidan has been reported as an antiviral (135) and antioxidant (158) agent. All research conducted on fucoidan have proved it to be non-allergenic with no significant toxicological effects in human and animals. Human trials involving orally administration of fucoidan to healthy volunteers showed no adverse effect on liver, renal and haematopoietic function over a period of three months (128).

1.3.5 Fucoidan's Anti-Cancer Potential

The anti-cancer property of fucoidan has been demonstrated *in vivo* and *in vitro* in different types of cancers. Nevertheless, it has been rarely used for its anti-cancer activity in clinical trials. Fucoidan mediates its activity through various mechanisms such as induction of cell cycle arrest, apoptosis and immune system activation. Additional activities of fucoidan have been reported that may be linked to the observed anti-cancer properties and these include induction of inflammation through immune system, oxidative stresses and stem cell mobilisation (159).

1.3.5.1 Fucoidan and Cell Cycle

Fucoidan induces cell death through modulation of cell cycle and results in accumulation of sub-G0/G1 cells (dead cells/apoptotic cells) in a variety of cell types (155, 160). It can also induce cell cycle arrest in other phases; Riou *et al*. (161) and Mourea *et al*. (162) reported arrest in G1 phase in a chemo-resistant non-small-cell bronchopulmonary carcinoma line by fucoidan from *Ascophyllum nodosum* and *Bifurcaria bifurcate*, respectively.

In an investigation of its mechanism of action, fucoidan demonstrated significant down regulation of cyclin D1, cyclin D2 and CDK4 in cancer cells (163-165). The crude fucoidan from *Fucus vesiculosus* increased the level of p21/WAF1/CIP1 in human prostate cancer PC3 cells and down-regulated E2F; a transcription factor that controls progression of cells from G1 to S phase (163).

In a recent study, fucoidan down-regulated cyclin E, CDK2, CDK4 resulting in G0/G1 arrest in human bladder cancer 5637 cells. Furthermore, immunoprecipitation assays revealed a significant increase in the binding of p21/WAF1/CIP1 to CDK2 and CDK4 in cells treated with fucoidan, suggesting that the induced G0/G1 arrest is due to suppression of CDK activity following direct binding of this CDK inhibitor to CDKs 2 and 4 (166). Table 1.4 summarises findings of studies examining the effects of fucoidan on cell cycle.

1.3.5.2 Fucoidan and the Apoptosis Pathway

Several studies examining a variety of cancers such as haematopoietic, lung, breast and colon cancers have shown that fucoidan-mediated cell death occurs through triggering apoptosis (Table 1.4) (160, 167, 168). A very low dose of fucoidan from *F. vesiculosus* (20 µg/mL) activated common caspases 3 and 7 in human colon cancer cells (167), whereas it induced the same activity in T-cell leukaemia at a much higher concentration (3 mg/mL) (165). Caspase 8 and 9 are also activated by fucoidan (167). Yamasaki-Miyamoto *et al.* showed that pre-treatment with caspase 8 inhibitor completely blocked fucoidan mediated apoptosis in MCF-7 breast cancer cell line (168). In contrast, in Zhang *et al*. study (160), the mediated apoptosis by fucoidan from *Cladosiphon okamuranus* in MCF-7 human breast cancer cell line was shown to be caspase independent. As cytochrome C and apoptosis inducing factor (AIF) increased in

Table 1.4. Effects of fucoidan on cell cycle and apoptosis molecules

Ref	Cell Type	Fucoidan Source	Dose (µg/mL)	Effects on Cell Cycle	Effects on Apoptosis Pathways		
					Extrinsic	Intrinsic	Common
(155)	lymphoma HS-sultan cells	*F. vesiculosus*	100	• ↑ sub G0/G1 • No G0/G1 or G2/M arrest	-	• ↓ MMP	• Caspase 3 activation
(165)	HTLV-1 infected T-cell HUT-102-cells	*C. okamurans*	3000	• G1 arrest • ↓ cyclin D2, c-myc • No changes in p21,p53	Apoptosis was reversed by caspase 8 inhibitor	• Caspase 9 activation • No changes in Bcl-2 and Bcl-XL • ↓survivin, cIAP-2	• Apoptosis was reversed by caspase 3 inhibitor
(169)	Hepatocellular carcinoma	*Okinawa mozuku*	22.5	• ↑ G2/M phase in HAK-1A, KYN-2, KYN-3 cell lines	-	• No clear caspase 9 activation in HAK-1B cell line	• No clear caspase 3 activation in HAK-1B cells
(168)	Breast cancer MCF7 cells	Not mentioned	1000	• ↑ sub-G1 fraction	• Caspase 8 activation	• Caspase 9 activation • ↓ Bid, cytosolic Bax, ↑ Bax, cytochrome C	• Caspase 7 activation • PARP cleavage
(170)	Acute leukaemia NB4 and HL-60 cells	*F. vesiculosus*	150	• ↑ sub-G1 fraction	• Caspase 8 activation	• caspase 9 activation • No changes in Bcl-2 or Bax. • ↓ Mcl-1, ↑ cytochrome C	• PARP cleavage • Caspase 3 activation
(167)	Colon cancer HT-29 and HCT116 cells	*F. vesiculosus*	20	-	• Caspase 8 activation • ↑ Fas, DR5, TRAIL • No significant effects on FasL and DR4	• Caspase 9 activation • ↑ cytochrome C, Smac/Diablo, Bak, t-Bid • No changes in Bcl-2, Bcl-xL, Bax, Bad, Bim, Bik, ↓ XIAP, survivin	• PARP cleavage • Caspase 3 and 7 activation
(171)	Lung cancer A549 cells	*U. pinnatifida*	50, 100, 200	• ↑ Sub-G1frction	-	• Caspase-9 activation • ↓ Bcl-2 , ↑ Bax	•↓ procaspase-3 •PARP cleavage
(160)	Human breast cancer MCF-7 cells	*Cladosiphon novae-caledoniae*	82, 410, 820	• ↑Sub-G1 • No significant changes in cell cycle distribution	• No changes in caspase-8	• Mitochondrial dysfunction • AIF and cytochrome C release • No cleavage of caspase-9 and Bid. • ↓ Bcl-2, Bcl-xl ,↑ Bax, Bad	• No activation of PARP and caspase-7 • All caspase inhibitors failed to attenuate FE-induced apoptosis
(172)	Hela cells	*Sargassum filipendula*	1500	-	-	• No effect on caspase 9 activation • ↑ cytosol AIF	• No effect on caspase 3 (Caspase independent)
(164)	Breast cancer MCF-7 cells	*F. vesiculosus*	400, 800, 1000	• G1 phase arrest • ↑ Sub G0/G1 ↓ cyclin D1 and CDK-4 gene expression	• Caspase-8 activation	• ↓ Bcl-2, ↑ Bax • Release of cytochrome C and APAf-1	• Caspase-dependent pathway
(163)	Prostate cancer PC-3 cells	*U. pinnatifida*	100	• G0/G1 phase arrest • ↓ E2F-1, ↑ p21Cip1/Waf	• DR5, caspase-8 activation	• ↓ Bcl-2 , ↑ Bax, • Caspase 9 activation	• Caspase-3 activation • PARP cleavage
(173)	Hepatocellular Carcinoma SMMC-7721 cells	*U. pinnatifida*	1000	• Non-significant accumulation in S-phase	• Caspase-8 activation	• Caspase-9 activation • MMP dissipation, Cytochrome C release • ↓ Bcl-2, ↑ Bax • ↓ *XIAP*, *livin* mRNA expression	• Caspase-3 activation
(166)	Human bladder carcinoma 5637 and T-24 cells	*F. vesiculosus*	100	• ↑ G1-phase, p21WAF1 • ↓ Cyclin E, D1, DK2, CDK4 • No change in p27KIP,p53 • ↑ p21WAF1 and CDK4 binding	-	-	-

the cytosol, it was concluded that fucoidan performed its activity through mechanisms altering mitochondrial function.

Fucoidan also affects other components of extrinsic and intrinsic pathways. Analysing the extrinsic pathway, 20 µg/mL crude fucoidan from *F. vesiculosus* increased the levels of the death receptors Fas, DR5 and TRAIL but not FasL and DR4 in human colon cancer cell lines (167). Contradictory results have been described in the expression of Bcl-2 family members in response to fucoidan (Table 1.4). Treatment of MDA-MB231 breast cancer cells with 820 µg/mL of low molecular weight (LMW) fucoidan resulted in a significant decrease in anti-apoptotic proteins Bcl-2, Bcl-xl and Mcl-1 (174). In contrast, no changes in expression of Bcl-2, Bcl-xl, Bad, Bim and Bik were observed in colon cancer cells when they were treated with 20 µg/mL fucoidan from *Fucus vesiculosus* (167). Taken together, these findings suggest that fucoidan may interact with several components of the apoptosis pathway.

1.3.5.3 Fucoidan and Angiogenesis

Fucoidan inhibits the formation of new vessels by which tumour cells receive their oxygen and required nutrients. Fucoidan inhibited the binding of VEGF, a key angiogenesis promoting molecule, to its cell membrane receptor (175). Xue *et al.* examined the anti-angiogenic properties of fucoidan in 4T1 mouse breast cancer cells both *in vitro* and *in vivo* and observed a significant dose-dependent decrease in VEGF expression in cells treated with fucoidan (176). Further, in a mouse breast cancer model using 4T1 cells, intraperitoneal injections of 10 mg/kg body weight fucoidan from *F. vesiculosus* markedly reduced the number of microvessels.

1.3.5.4 Fucoidan and Metastasis

Fucoidan significantly decreased tumour cell metastasis to the lungs in animals that were intravenously injected with rat mammary adenocarcinoma 13762 MAT cells (177). It was first reported that fucoidan inhibits cell invasion through competing with tumour cell binding with laminin in the basement membrane (178). Subsequent studies then revealed that fucoidan binds to fibronectin with high affinity and prevents attachment of tumour cells (179).

Tumour invasion requires the secretion of proteolytic enzymes by tumour cells to break down the extracellular matrix (ECM) proteins (e.g. collagen, fibronectin and laminin), with the matrix metalloproteinases (MMPs) MMP-2 and MMP-9 playing a major role. Fucoidan attenuates both expression and activity of these enzymes (180).

Selectin inhibition by fucoidan interferes with tumour cell–platelet interaction. When highly metastatic MDA-MB-231 breast cancer cells were plated in platelet-coated plates in the presence or absence of 100 µg/mL fucoidan the number of cells attached to the platelets decreased by 80% in the presence of fucoidan (181). Interaction of tumour cells with platelets is one of the key factors in facilitating the early steps of tumour cell migration. During tumour cell migration, most circulating tumour cells do not survive attack from immune cells or the shear forces of the blood stream. However, they can attach to platelets to induce platelet aggregation allowing the tumour cell cluster to survive in the micro-vascular system. It was concluded that fucoidan inhibited P-selectin residing on the platelet surface and led to reduced number of attached tumour cells. Fucoidan can also inhibit other adhesion molecules such as integrins residing on the tumour cell surface and can modify distribution of their subunits.

1.3.5.5 Fucoidan and Signalling Pathways

The Ras/Raf/MAPK pathway is often hyperphosphorylated in a variety of human cancers. Various studies have shown that fucoidan inhibits tumour cell proliferation by decreasing ERKs activity through reduction of its phosphorylation (155, 182).

JNK and p38 are other MAPK superfamily members whose activity is altered by fucoidan. Fucoidan induced cell death in breast cancer cells through phosphorylation and of JNK and p38 after 30 minutes. The fucoidan-induced apoptosis significantly annulled in the presence of JNK inhibitor, indicating critical role of JNK in fucoidan-mediated apoptosis (160).

Similarly, the PI3K/Akt, GSK and Wnt pathways have been shown to be triggered by fucoidan. Akt over-activation is linked with drug resistance and tumour cell survival. Most of the studies have reported inactivation of Akt by fucoidan. PI3k, an upstream molecule of Akt, is also inhibited by fucoidan (180). Upregulation of the Wnt signalling pathway is believed to have a critical role in prostate cancer development, survival and progression. Fucoidan from *F.*

vesiculosus activated GSK-3β in PC3 human prostate cancer cells resulting in hypo-phosphorylation and inactivation of β-catenin (163).

1.3.5.6 Fucoidan and the Immune System

The effects of fucoidan on molecules of the immune system have been studied both *in-vitro* and *in-vivo* and effects on both cellular and humoral elements have been described. Fucoidan increases both activity and number of NK cells *in vivo* (183, 184). Increase in the number of cytotoxic T-cells (CTLs) has also been reported. A high-molecular-weight (HMW) fucoidan from *Cladosiphon okamuranus* (200–300 KDa) induced a large increase in the proportion of murine cytotoxic T cells (185). Investigation of the role of fucoidan on dendritic cell (DC)-mediated T-cell cytotoxicity has revealed that the stimulation of CTLs was more effective in fucoidan-treated DCs as CTLs co-cultured with fucoidan-treated DCs exerted a high level of specific lysis of breast cancer cells (186).

In a recent study, the role of fucoidan in DCs function and its adjuvant effect have been examined *in vivo*. Fucoidan was systemically administrated to mice by intraperitoneal injection. Examination of the spleen DCs revealed up-regulation of maturation markers as well as production of IL-6, IL-12 and TNF-α. Fucoidan was then used as an adjuvant *in vivo* with ovalbumin antigen and induced Th1 mediated immune response and CTL activation (187).

1.3.5.7 Fucoidan and Malignant Transformation *in Vitro* and *in Vivo*

Few studies have reported the potential of fucoidan to inhibit neoplastic transformation. Teas *et al*. fed rats with dietary seaweed (*Laminira*) for 55 days and administrated the carcinogen 7,12-dimethylbenz(a) anthracene intragastrically. Following 26 weeks monitoring, experimental rats showed a significant delay in the median time for tumour appearance (19 vs 11 weeks in the control group) (188).

Transforming growth factor β1 (TGFβ1) is believed to promote tumour development and metastasis through epithelial to mesenchymal transition (EMT), a process that enables epithelial cells migrate to distant areas during late stages of breast cancer development (189). To trigger tumour progression, TGFβ1 recruits TGF receptors (TGFR) residing on the cell surface. The investigations of effects of fucoidan on TGFβ1-promoted carcinogenesis in MDA-

MB-231 breast cancer cells have indicated that fucoidan decreased the expression of TGFRs and affected the downstream signalling molecules which are involved in TGFβ1-mediated EMT (190).

Epidermal growth factor (EGF) is another carcinogenesis promoter, which induces tumour transformation through overexpression and activation of EGF receptor (EGFR). EGFR has a key role in cell proliferation and differentiation and many carcinomas arise from its mutations (191). Lee *et al.* examined the role of fucoidan on the activation of EGFR and EGF-mediated neoplastic transformation (192). They utilised murine JB6 Cl41 epidermal cells and induced cell transformation by EGF in the presence of fucoidan from *L. guryanovae*. Fucoidan markedly reduced the EGFR activation through hypo-phosphorylation. It also inhibited EGF-tumourigenic activity through inhibition of AP-1, a transcription factor responsible for cell proliferation regulation.

1.3.5.8 Fucoidan as a Synergistic Anti-Cancer Agent

The ability of fucoidan to synergise with standard anti-cancer agents and/or reduce toxicity has recently been investigated. Ikeguchi *et al.* examined the synergistic effect of a HMW fucoidan with colorectal cancer chemotherapy agents; oxaliplatin plus 5-fluorouracil/leucovorin (FOLFOX) or irinotecan plus 5-fluorouracil/leucovorin (FOLFIRI). The test patients received 150 mL/day for 6 months of liquid that contained 4.05 g fucoidan. From the commencement of chemotherapy, toxicities and chemotherapy efficiency were compared. Fucoidan showed no side effects such as allergic dermatitis. Diarrhoea, neurotoxicity and myelo-suppression were not suppressed by fucoidan, whereas general fatigue was significantly decreased from 60% to 10%. The patients were followed for approximately 15 months and the survival rate of the patients who received fucoidan was longer than that of the control participants; however the difference was not significant, probably due to the small numbers (193).

Fucoidan affects the migration and invasion of multiple myeloma (MM) cells treated with chemotherapy drug cytarabine. The human myeloma cell lines RPMI8226 and U266 were treated with crude fucoidan from *F. vesiculosus* for 72 hours and then cytarabine for 6 hours. Fucoidan reduced cell migration and down-regulated expression of CXCR4 and MMP-9 (194). Fucoidan from *Saccharina cichorioides* has been reported to synergise with the anti-tumour

activity of low dose resveratrol on invasive and highly motile HCT 116 colon cancer cell line (195). In the colony formation assay, fucoidan plus resveratrol reduced the colony number by 60% compared to 34% and 27% in resveratrol alone or fucoidan alone, respectively.

Zhang *et al*. studied the combinatory effect of fucoidan and three commonly used anti-cancer agents; cis-platin (CDDP), tamoxifen (TAM) and paclitaxel (Taxol) on signal transduction pathways. Fucoidan from *Cladosiphon navae-caledoniae* plus anti-cancer agents reduced the ERK phosphorylation in MDA-MB-231 breast cancer cells compared to untreated control or fucoidan alone (196). Dietary fucoidan synergistically reduced cell growth in the OE33 cell line when it was combined with lapatinib, a targeted therapy that acts as a tyrosine kinase inhibitor in advanced HER2-positive breast cancer cells (197).

1.4 Research Aims

The focus of this PhD project was to investigate the biological anti-tumour activities of the alga derived polysaccharide, fucoidan, on acute myeloid leukaemia cells. It was hypothesised that fucoidan exerts anti-tumour activity in AML cells and augments the efficacy of standard AML therapy.

This study aimed to assess the inhibitory role of fucoidan on AML cell proliferation *in vitro* and *in vivo*, and to investigate the effect of fucoidan when combined with standard chemotherapeutic regimens in inhibition of AML cell growth *in vitro* and *in vivo*. The specific aims of this project were to:

1. Determine if fucoidan has cytotoxic activity against AML cell lines, *in vitro*

2. Characterise the mechanisms by which fucoidan induces cell death in AML cells

3. Identify if fucoidan has anti-tumour property as a prophylactic agent during AML development, *in vivo*, and to characterise the underlying mechanisms

4. Determine the synergistic effects of fucoidan with standard chemotherapy regimens for AML, both *in vitro* and *in vivo*

CHAPTER TWO

Materials and Methods

2.1 Cell Culture

2.1.1 Cell Line Characteristics

2.1.1.1 HL60 Cell Line

HL60 is a human acute promyelocytic leukaemia (APL) cell line (198). Morphologically, these cells display a myeloblastic/promyelocytic feature; large, basophilic cytoplasm containing azurophilic granules and rounded nuclei with 2-4 distinct nucleoli. The HL60 cells are negative for translocation t(15;17) which is the karyotypic marker of most cases of APL in humans. In immunophenotyping analysis, the majority of HL60 cells are positive for CD13 (the pan-myeloid marker), CD15 (granulocytes-associated antigen), CD11a (Integrin) and CD44 (hyaluronic acid receptor). These cells are non-adherent and grow in a suspension culture with a doubling time varying between 24 to 40 hours.

2.1.1.2 NB4 Cell Line

NB4 is a human APL cell line which unlike HL60 carries the specific chromosomal translocation t(15;17) (199). Morphologically, NB4 cells are abnormal large promyelocytes with hyper-granular cytoplasm and prominent nuclei. In analysis of surface markers, NB4 cells are positive for CD13, CD15, and CD44 and also show low expression of CD11b. The NB4 cells grow in a suspension culture with a doubling time of 24-36 hours.

2.1.1.3 K562 Cell Line

K562 is an erythroleukaemia type cell line derived from a patient with chronic myelogenous leukaemia in blastic crisis (200). Morphologically, K562 cells are lymphoblastic; round small cells with large round nuclei and high nuclear to cytoplasm (N/C) ratio. These cells are non-adherent and their doubling time is around 24 hours.

2.1.1.4 KG-1a Cell Line

KG-1a is a human undifferentiated myeloblastic leukaemia and is a sub-clone of the KG-1 cell line (201). KG-1a represents an early or primitive stage of development and is generally

irresponsive to colony stimulating factor and is unable to differentiate into further mature cells. Morphologically, KG-1a cells are small round cells with presence of 1-2% giant myeloblasts. These cells are leukaemia stem cell-like cells and highly express CD34 while are negative for CD38. KG-1a grows slowly with doubling time of approximately 50 hours.

2.1.1.5 Kasumi-1 Cell Line

Kasumi-1 cells are human acute myeloblastic leukaemia cells containing translocation t(8;21) representative of AML-M2 in the FAB classification (202). Morphologically, these cells show myeloid maturation; the myeloblasts are the major cells with variation in size and N/C ratio. Kasumi cells express CD13, CD33 and are negative for CD34. The doubling time is around 40-45 hours.

2.1.1.6 Yac-1 Cell Line

Yac-1 cell line is a mouse T-cell lymphoma which was induced by inoculation of Moloney leukaemia virus into a new-born mouse (203). Morphologically, Yac-1 cells are small round to oval lymphoblasts and can form clusters in suspension. The most important feature of these cells is their sensitivity to the cytolytic function of NK-cells. The non-adherent Yac-1 cells grow rapidly and their doubling time is around 20 hours.

2.1.2 Cell Culture

The K562 cell line was purchased from Sigma-Aldrich. The other cell lines were stored in liquid nitrogen at Menzies Institute for Medical Research. The cells were thawed and used for experiments when they reached log phase. The cells were cultured in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (JRH Biosciences, Australia), 2 mM L-glutamine and 1% penicillin/streptomycin solution (5000 U/mL penicillin G and 5000 µg/mL streptomycin, Sigma Aldrich, U.S.A). For the Kasumi-1 cell line, the RPMI-1640 medium was supplemented with 20% heat inactivated fetal calf serum.

2.1.2.1 Thawing Cell Lines

Cell line vials were removed from either the -80°C freezer or vapour phase of liquid nitrogen and thawed in a water bath at 37°C. The thawed cells were transferred to a 15 mL BD Falcon™ conical tube (Becton, Dickinson and Company Biosciences, U.S.A) and 10 mL media was gradually added to cells. After centrifugation (500 g for 5 minutes), 10 mL of fresh medium was added and the cells were transferred to cell culture flasks (Becton, Dickinson and Company Biosciences, U.S.A) and incubated at 37°C with 5% CO_2.

2.1.2.2 Culturing Cell Lines

Cells were cultured in either 25 cm^2 or 75 cm^2 BD Falcon™ cell culture flasks. Cells were counted using a haemocytometer using trypan blue exclusion to assay viability. Cells were maintained at a concentration between 1×10^5 and 1×10^6 cells/ mL by sub-culturing the cells every 2-3 days.

2.1.2.3 Cryopreservation of Cell Lines

Cells were collected by centrifugation at 500 g for 5 minutes and resuspended in media at 1×10^7 cells/ mL. Equal volume of media containing 20% DMSO was gradually added to the cell suspension. Aliquots (1 mL) were transferred to CryoTubes. Tubes were placed in a -80 °C freezer in a box insulated with cotton wool to slow freezing, and then transferred to the vapour-phase of liquid nitrogen for storage.

2.2 Cell Treatments

Cell lines were treated with the following reagents for variable doses and times as indicated in Table 2.1.

Table 2.1 Cell treatments

Reagents	Stock Preparation	Treatment
Purified fucoidan from *Undaria pinnatifida* (Marinova Pty Ltd)	10 mg/mL in PBS (filtered through a 0.21-µm syringe filter (Millipore)	Variable doses for variable times as indicated.
Purified fucoidan from *Fucus vesiculosus* (Marinova Pty Ltd)	10 mg/mL in PBS (filtered through a 0.21-µm syringe filter (Millipore)	Variable doses for variable times as indicated.
Fucoidan from *Fucus vesiculosus* (Sigma-Aldrich)*	10 mg/mL in PBS (filtered through a 0.21-µm syringe filter (Millipore)	Variable doses for variable times as indicated.
Phorbol Myristate Acetate (PMA, Boehringer Mannheim, Australia)	1 mg/mL in DMSO	20 ng/mL for 48 hours.
Pan-Caspase Inhibitor Z-VAD-FMK (Abcam)	10 mM in DMSO	40 µM for 1 hour.
Arsenic Trioxide (ATO, Sigma-Aldrich)	Stock: 10 mM in 1.65 M NaOH. Working solution: 100 µM in PBS.	0.25, 0.5 and 1 µM for variable times as indicated.
All-Trans Retinoic Acid (ATRA, Sigma-Aldrich)	Stock: 10 mM in absolute ethanol. Working solution: 100 µM in PBS.	0.25, 0.5 and 1 µM for variable times as indicated.

* The endotoxin level of the crude fucoidan solution (Sigma-Aldrich) was detected using chromogenic LAL endpoint assay (GenScript, U.S.A).

2.3 DNA Content Analysis (Cell Cycle Assay)

2.3.1 Reagents

2.3.1.1 Propidium Iodide (PI) Solution

PI powder was purchased from Sigma-Aldrich, U.S.A (Cat. No. P4170). The powder was dissolved in PBS to a concentration of 1 mg/mL and stored at 4 °C, protected from light.

2.3.1.2 RNase A solution

DNAase-free RNase A solution (100 mg/mL; 7000 units/mL) was purchased from Qiagen (Cat. No. 194101). A 10 mg/mL RNase A working solution was freshly prepared by diluting the RNase A solution in PBS.

2.3.1.3 DNA Content Staining Solution

The DNA content staining solution was made freshly. For each sample, 40 µL of PI solution (see 2.3.1.1) and 10 µL of prepared RNase A (see 2.3.1.2) were added to 950 µL of PBS.

2.3.2 DNA Content Analysis (Cell Cycle Assay)

Evaluation of the cell cycle phase distribution and sub-G0/G1 population was performed using propidium iodide staining as described previously (204). In brief, approximately 1-2 × 10^6 cells were harvested following treatment as indicated, pelleted (500 g for 5 minutes) and washed in cold PBS. Cells were resuspended in 500 µL cold PBS and put on ice. To fix the cells, 4 mL of ice-cold 70% ethanol was added to cells drop wise with mixing. Cells were stored at -20°C overnight.

The ethanol was aspirated after centrifugation (500 g for 10 minutes) and cells were washed once in PBS (500 g for 5 minutes). The cells were resuspended in 1 mL DNA content staining solution, transferred into 12×75 mm Polystyrene test tubes (BD Company, Cat. No. 352052) and incubated at room temperature for 30 minutes in the dark. Cell cycle was analysed using the FACSCanto^{TM}II flow cytometer (BD Biosciences, U.S.A). The data was analysed by Kaluza Software (BD Biosciences, U.S.A).

2.4 WST-8 Cell Proliferation Assay

Cell proliferation was assessed using water-soluble tetrazolium salt WST-8 (Sigma-Aldrich). The assay is based on the extracellular reduction of WST-8 (205) by the glycolytic NAD(P)H production of viable cells. The WST-8 reduction produces yellow-colour formazan dye and generates colour which is directly proportional to the number of the living cells. According to the manufacturer's instructions, 10 µL of WST-8 solution was added to 100 µL treated cells in a 96-well Corning® cell culture micro-culture plate for the last 4 hours of incubation. Finally, the absorbance was determined at 450 nm by spectrophotometry (SpectraMax® Plus^{384}, Molecular Devices, U.S.A).

2.5 DNA Fragmentation TUNEL Assay

The presence of fragmented DNA was detected using the Apo-BrdU TUNEL apoptosis assay kit (Invitrogen Ltd) according to the manufacturer's protocol. In brief, 1-2 × 10^6 cells were harvested following treatment as indicated, pelleted (500 g for 5 minutes) and washed with PBS and resuspended in 0.5 mL PBS. Paraformaldehyde (5 mL at 1%) was added to the suspension and tubes were put on ice for 15 minutes. To remove the paraformaldehyde, cells were centrifuged (500 g for 5 minutes) and washed twice with 5 mL PBS. The cell pellet was resuspended in 0.5 mL PBS. Five mL of 70% ethanol was added and cells were stored for 18 hours in a -20°C freezer. After 18 hours, the experimental samples in addition to a 1 mL aliquot of manufacturer-supplied positive and negative control cells (containing approximately 1 × 10^6 cells) were centrifuged at 500 g for 5 minutes and the ethanol was removed by aspiration. Cells were washed twice with wash buffer provided by the kit. The cell pellet was resuspended in 50 µL of DNA-labelling solution (10 µL of reaction buffer, 0.75 µL of terminal deoxynucleotidyl transferase, 8.0 µL of 5-Bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) and 31.25 µL of Milli-Q water) and incubated at 37°C on heat-block for 60 minutes.

Following the incubation time, 1 mL of provided rinse buffer was added to each tube and cells were centrifuged (500 g for 5 minutes). This step was repeated and then 100 µL antibody staining solution containing Alexa Fluor® 488 dye-labeled anti-BrdU antibody was added to the cell pellet and cells were incubated at room temperature for 30 minutes. Finally, 500 µL PBS was added to the tubes and the presence of DNA fragmentation was analysed by FACSCanto^{TM}II flow cytometer (BD Biosciences).

Note 1. The peak emission of Alexa Fluor® 488 is 519 nm and was measured in the FL-1 channel.

2.6 Annexin V/PI Apoptosis Detection Assay

2.6.1 Reagents

Annexin V binding buffer and annexin V-FITC antibody were purchased from Abcam. To prepare the 1X annexin V binding buffer, the 10X annexin V binding buffer was diluted in

PBS. To prepare the 1X annexin V-FITC antibody, the 5X annexin V-FITC antibody was diluted in 1X annexin V binding buffer.

2.6.2 Annexin V/PI Apoptosis Detection Assay

The presence of early and late apoptosis was detected using annexin V/PI apoptosis assay as described previously (206). Briefly, 1×10^5 cells were harvested following treatment as indicated, pelleted (500 g for 5 minutes) and washed with PBS and resuspended in 500 µL of 1X annexin V binding buffer. Five µL of 1X annexin V-FITC antibody was added to the cell suspension and tubes were incubated at room temperature for 10 minutes. Propidium iodide solution (2.3.1.1) was added to each sample (1 µL) and cells were incubated at room temperature for an extra 5 minutes. The presence of different stages of apoptosis was analysed by FACSCanto^{TM}II flow cytometer (BD Biosciences) and data was analysed using Kaluza Software (BD Biosciences, U.S.A)

2.7 Western Blot Analysis of Cytoplasmic and Nuclear Proteins

2.7.1 Preparation of Cytoplasmic and Nuclear Protein Extracts

For this protocol, 25 mL cells at 5×10^5 cells/mL (i.e. 1.25×10^7 cells total) were used. Additional sample tubes and buffers were pre-cooled for over an hour prior to extraction. Nuclear and cytoplasmic extracts were prepared from a modified method (207). Briefly, cells were harvested following treatment as indicated, pelleted (500 g for 5 minutes at 4 °C) and washed twice in 10 mL of ice-cold PBS. The resulting cell pellet was resuspended in 750 µL of lysis buffer (containing 10 mM NaCl, 10 mM Tris (pH 7.4), 3 mM MgCl2, 0.1 mM EDTA (pH 8.0), 0.5% Igepal) and incubated on ice for 5 minutes to lyse the cells. Following centrifugation (500 g for 5 minutes at 4 ºC) the supernatant containing the cytoplasmic protein fraction was transferred to a fresh tube and placed on ice. The remaining nuclei pellet was washed in 750 µL of lysis buffer without Igepal (containing 10 mM NaCl, 10 mM Tris (pH 7.4), 3 mM MgCl2, 0.2 mM EDTA (pH 8.0)) and centrifuged at 500 g for 5 minutes and 4 ºC. The nuclei were then resuspended in 25 µL of nuclei lysis buffer (containing 400 mM NaCl, 7.5 mM MgCl2, 0.1 mM EDTA (pH 8.0), 1 mM DTT) and 1 µL of protease inhibitor

(containing aprotinin, leupeptin, complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science, Switzerland)) to be incubated for 15 minutes on ice with shaking ensuring nuclear lysis. The suspension was centrifuged at 15,000 g for 5 minutes and 4 ºC and supernatant containing the nuclear proteins was transferred to a fresh tube. Protein concentration was measured using Bradford assay (208) (see Appendix B).

2.7.2 SDS-PAGE and Western Blotting

To separate proteins according to their molecular weight, SDS-PAGE (sodium dodecyl sulphate-poly-acrylamide gel electrophoresis) was performed. Briefly, protein extracts (10-20 µg) were combined with an equal volume of 2X sample loading buffer (10 µL containing: 120 mM Tris HCl (pH 6.8), 20% glycerol, 4% SDS, 0.04% Bromophenol blue, 5% β-mercaptoethanol) and heated at 95 ºC for 5 minutes on a heat block. Spectra™ Multicolor Broad Range Protein ladder (10-260 kDa) (ThermoScientific, U.S.A) and 20 µL of samples were carefully loaded on the 12% Mini-PROTEAN® TGX™ pre-cast gel (Bio-Rad, U.S.A) with sufficient SDS-PAGE running buffer (192 mM glycine, 25 mM Tris (pH 8.0), 0.1% sodium dodecyl sulphate). Samples were initially electrophoresed for 15 minutes at 100 V and were subsequently subjected to 150 V until completion.

The separated proteins were transferred from gel onto a 0.45 µM nitrocellulose membrane (Bio-Rad, U.S.A) using a wet transfer. The transfer assembly (consisted of the gel and membrane in direct contact surrounded by two layers of filter paper (Whatman, U.S.A) and a support pad on either side pressed together by the support grid) was placed in the transfer apparatus, immersed in western transfer buffer (20mM Tris (pH 8.3, 150 mM glycine, 20% methanol) and transfer was facilitated by 100 V for 90 minutes at 4 °C.

2.7.3 Protein Detection

The membrane was placed in blocking reagent (Blocking reagent (Roche Applied Sciences, Switzerland) diluted 1:10 in 1X TNT (10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween) and incubated at room temperature for 1 hour with shaking. The membrane was incubated with a specific dilution of primary antibody (see Appendix A) overnight at 4 ºC followed by three

10-minute washes with 1X TNT at room temperature with shaking. The membrane was incubated in 5 mL of secondary antibody (see Appendix A) for 45 minutes at room temperature followed by three 10-minute washes with 1X TNT at room temperature with shaking.

To visualise the membrane, it was incubated with a mixture of 1 mL of SuperSignal® West Pico Stable Peroxide Solution and 1 mL of Pico Luminol/Enhancer solution (ThermoScientific, U.S.A) for 5-10 minutes with shaking in minimal light. Excess chemiluminescent complex was removed and the membrane was exposed for adequate lengths of time up to 10 minutes using the Chemi-Smart 5000 (Vilber Lourmat, France).

2.8 Analysis of Fucoidan

The constituents of fucoidan was determined in the laboratory of Marinova Pty Ltd. These experiments were performed by spectrophotometric analysis of fucoidan as follows: Total carbohydrate content was determined by spectrophotometric analysis of the hydrolysed compound in the presence of phenol, based on a method described by Dubois *et al*. (209). Sulphate content was analysed spectrophotometrically using a $BaSO_4$ precipitation method ($BaCl_2$ in gelatin), based on the work of Dodgson (210, 211). Analysis of polyphenol content was also determined spectrophotometrically, using a method based on the Folin-Ciocalteu reagent (phosphomolybdate/phosphotungstate) (212-214). The carbohydrate profile was determined using a GC-based method for the accurate determination of individual monosaccharide ratios in a sample. This method relies on the preparation of acetylated alditol derivatives of the hydrolysed samples (215). Molecular weight profiles were determined by Gel Permeation Chromatography, with the aid of a Size-Exclusion Column, and were reported relative to Dextran standards.

2.9 *In Vivo* Experiments

2.9.1 Animals

A total number of 120 athymic Balb/c nude mice (nu/nu) were obtained from the Cambridge Facility Farm (CFF) and housed at the animal facility, Medical Sciences Building 2, Menzies Institute for Medical Research. All animal studies were approved by the University of Tasmania Animal Ethics Committee (reference number: A13940) and were performed in accordance with the institutional guidelines.

2.9.2 Mice Identification System

The ear punch system was used for mice identification (Table 2.2) and a maximum of 5 mice with different ear punches were placed in one cage. A unique identification code was also prepared by the CFF for each mouse. To identify different cages, cage cards were used. The following information was included on each card: species, strain, mice identification code, ear punch category, date of birth, sex, date of arrival and names and contact details of responsible investigators.

Table 2.2 Mice identification by ear punch

Ear Punch Category	Description
1R	1 punch in right ear
1L	1 punch in left ear
1R1L	1 punch in right ear and 1 punch in left ear
2R	2 punches in right ear
2L	2 punches in left ear
NM	Non-marked

2.9.3 Mice Health Monitoring

A monitoring sheet was prepared for each experiment (see Appendix C) and all animals were checked daily. Changes in behaviour, food or water consumption, aggression, muscular rigidity and reaction to handling were recorded. Mice were weighed daily and the weight was recorded.

2.9.4 Animal Treatment

Mice were treated with different substances as indicated in Table 2.3. As shown, various injection routes including intraperitoneal (IP), Sub-cutaneous (SC) and intravenous (IV) were used in the experiments. The following general requirements were applied: 1. the pH of solutions injected was verified to be 7.3 to 7.45; 2. isotonic sterilised solutions were warmed to body temperature when injecting either IV or IP; 3. where different substances were to be injected, both chemicals were combined in a single solution.

Table 2.3 Animal treatments

Substance	Route	Stock Preparation*
Fucoidan (Sigma-Aldrich)	Oral	70 mg/mL fucoidan in Milli-Q water
Fucoidan (Sigma-Aldrich)	IP	20 mg/mL fucoidan in sterile PBS
Fucoidan (Sigma-Aldrich)	IV	2 mg/mL fucoidan in sterile PBS
All-Trans Retinoic Acid (ATRA) (Sigma-Aldrich)	IP	A stock of 100 mM ATRA (30 mg/mL) was prepared by dissolving ATRA powder in DMSO.
Arsenic trioxide (ATO) (Sigma-Aldrich)	IP	A stock of 300 mM (59.34 mg/mL) ATO was prepared by dissolving ATO powder in 1.65 M NaOH.

*All stock solutions were filtered through a 0.21-µm syringe filter (Millipore). To prepare the solutions for injection, stock solutions were diluted in sterile PBS.

For S.C injection, the animal was restrained with the non-dominant hand. The syringe was held with the dominant hand with the bevel of the needle facing upwards. The needle was inserted

and the skin was gently tented upwards with the needle to confirm that the needle was in the subcutaneous space. The solution was injected and the plunger was depressed until the solution was fully administered. The animal was placed back into its cage and observed for any complications. For S.C injections, a needle of 26 gauge × 1/2 in. 0.45 mm × 13 mm (Becton Dickinson) was used. If the injection was successful, a small swelling under the skin was seen.

For IP injections, the animal was restrained with the non-dominant. The syringe was held with the dominant hand with the bevel of the needle facing upwards. The substance was injected into the lower right quadrant of the abdomen towards the head at a 30-40^{o} angle. The solution was injected and the plunger was depressed until the solution was fully administered. The animal was placed back into the cage and observed for any complications. For IP injection, a needle of 30 gauge × 1/2 in. (Becton Dickinson) was used.

For IV injection, the two lateral tail veins were used. The animal was restrained in a rodent cone-shaped restrainer. The tail was swabbed with 70% alcohol and rotated ¼ turn to place the tail veins dorsally for easier injection. The needle was approached at a 15-20° angle at the distal portion of the tail and inserted parallel to the tail vein penetrating 2-4 mm into the lumen while keeping the bevel of the needle face upwards and the material was injected slowly. A successful injection resulted in the material entering the vein with no resistance and blanching of the tail vein for the duration of the injection.

2.9.4.1 Oral Administration of Fucoidan

150 µg/g body weight (b.w.) fucoidan was orally administered. Mouse weight was measured daily and the required amount of fucoidan was mixed with peanut butter and a paste was prepared. For each mouse the peanut butter paste was prepared freshly just before feeding (Figure 2.1). The prepared fucoidan-peanut butter paste was put on a wooden applicator stick and put in the cage with a single mouse. Each mouse was not returned to its own cage until the entire paste was eaten. The control group was fed with peanut butter mixed with Milli-Q water in the same manner.

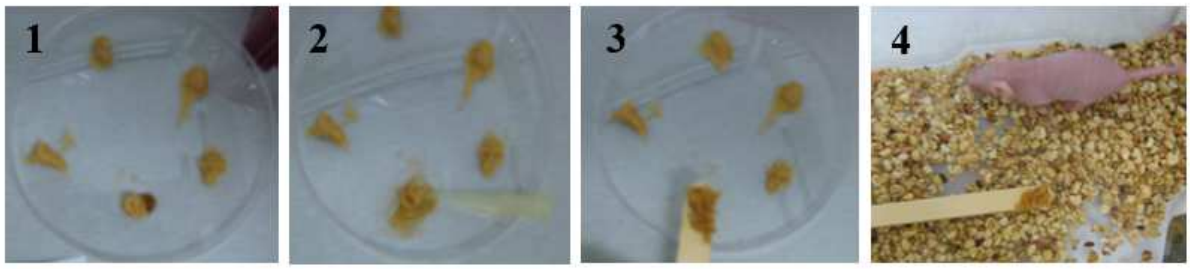


Figure 2.1. Preparation of fucoidan for oral administration. 1) The required amount of fucoidan was added to peanut butter. **2)** Fucoidan was mixed with peanut butter. **3)** The prepared paste was put on a wooden applicator stick. **4)** The fucoidan paste was put in the cage with a single mouse

2.9.4.2 Intraperitoneal Injection of Fucoidan

Either low dose of 30 µg/g b.w. or high dose of 100 µg/g b.w. of fucoidan was administered by injection according to the mice weight measured daily. An injection volume of 200 µL of freshly prepared solution was injected into the lower quadrant of abdomen as described (see Section 2.9.4).

2.9.4.3 Intravenous Injection of Fucoidan

One daily dose of 6 µg/g b.w. fucoidan was administered by injection according to the mice weight measured each day. An injection volume of 200 µL of freshly prepared solution was injected into the lower quadrant of the abdomen as described (see Section 2.9.4).

2.9.4.4 Intraperitoneal Injection of Arsenic Trioxide

One daily dose of 2.5 µg/g b.w. ATO was administrated by injection according to the mice weight measured each day. An injection volume of 200 µL of freshly prepared solution was injected into the lower quadrant of the abdomen as described (see Section 2.9.4).

2.9.4.5 Intraperitoneal Injection of ATRA

One dose of 1.5 µg/g b.w. ATRA was administrated three times a week by injection according to the mice weight measured each day. An injection volume of 200 µL of freshly prepared solution was injected into the lateral tail veins as described (see Section 2.9.4).

2.9.5 Establishment of Human Leukaemia Xenograft Models

Acute promyelocytic leukaemia NB4 cell line was cultured in RPMI as explained in 2.1.2.2. The medium was replaced with fresh RPMI medium 3 to 4 hours before harvesting. Cells were centrifuged at or below 500 g for 5 minutes and washed twice with 20 mL of sterile PBS free of serum. Cells were resuspended in sterile PBS at the final concentration of 2.5×10^7 cells/mL and transferred to sterile CryoVials. For each mouse, a volume of 200 µL of the prepared cell suspension containing 5×10^6 cells was injected. Cells were injected subcutaneously in the right flank of each mouse as described in 2.9.4. Mice injected with NB4 cells were monitored daily for tumour burden appearance.

2.9.6 Tumour Volume Measurement

Tumour volume was measured daily and the results were recorded in the monitoring sheet. The measuring surface was cleaned and dried. A flexible plastic ruler was sprayed with ethanol 70% and dried. The general restrain following by lifting the animal with the non-dominant hand was performed and the tumour location was faced toward the investigator. The length and width of the tumour was measured in millimeter (mm) three times and the mean value was recorded. To calculate the tumour volume, the below formula was used:

$$\text{Tumour Volume } (mm^3) = \frac{1}{2} \times (\text{length} \times \text{width}^2)$$

The tumour volume was recorded in the monitoring sheet (see Appendix C) and the experiment was terminated if the tumour size reached 1000 mm^3.

2.9.7 Animals Euthanasia

Animals were humanely sacrificed using carbon dioxide as per the AVMA Guidelines on Euthanasia 2013 (216).

2.9.8 Tumour Volume Doubling Time

The tumour volume doubling time and tumour growth rate were calculated by the algorithm provided by http://www.doubling-time.com (217).

2.9.9 Assessment of the Tumour Mass

2.9.9.1 Tumour Mass Cell Suspension Preparation

To remove the tumour mass, an incision was made in the area of the tumour using dissecting scissors and microscopic forceps (Met-App Co.) and the tumour mass was carefully removed. Fresh tissue sample was dissected and mixed with 2 mL PBS and filtered through a 40 µM sterile nylon mesh strainer (Fisher Scientific, Cat. No. 352340). The cell suspension was then washed with PBS (500 g for 5 minutes) and the cell pellet was resuspended in 2 mL sterile PBS.

2.9.9.2 Cell Surface Marker Analysis

The cell surface markers of the tumour mass removed from mice were analysed using various antibodies by flow cytometry. The prepared cell suspension (50 µL) was mixed with 5 µL of desired cell surface antibodies or the appropriate isotype negative controls in 12×75 mm polystyrene test tubes and incubated at 4 °C for 30 minutes. PBS (500 mL) containing 10% paraformaldehyde was added to the mixture, and the expression of cell markers was analysed with FACSCanto^{TM}II flow cytometer (BD Biosciences, U.S.A). The flow cytometry results were analysed with Kaluza Software (BD Biosciences, U.S.A).

2.9.10 Natural Killer Cell Activity

To analyse the cytolytic activity of mice splenic natural killer (NK) cells, the spleen mononuclear cells (MNCs) were used as effector cells and the NK sensitive Yac-1 cell line was used as target cell. The activity of NK cells in lysing these cells was measured with Cell Trace Violet and PI cytotoxicity assay using flow cytometry.

2.9.10.1 Preparation of Splenic Mononuclear Cells

Animals were sacrificed (see 2.9.8) and the mouse body was placed on its back and sprayed with 70% ethanol. A small incision was made in the left side of the peritoneum and the spleen was gently pulled and the connective tissues behind it were torn. The removed spleen was dissected and filtered through 40 µM sterile nylon mesh strainer (Fisher Scientific, Cat. No. 352340). To do this, the spleen pieces were placed on the strainer and mashed and minced using a syringe plunger firmly so the connective tissue remained on the top of the strainer and the cells were filtered through by adding PBS. The cell suspension was washed with PBS (500 g for 5 minutes) and resuspended in 5 mL of PBS.

To isolate the MNCs, the prepared splenic cell suspension was gently homogenised and slowly added to a tube previously loaded with 5 ml of Ficoll-paque®-1077 (Sigma Company, Cat. No. 10771). The tube was carefully loaded into the centrifuge buckets without disturbing the layers. The mixture was centrifuged (400 g, for 30 minutes) using slow acceleration and deceleration rates. After centrifugation, the following layers were visible in the tube from top to bottom: PBS, a layer of MNCs, Ficoll-Paque, and erythrocytes and poly-morphonuclear cells presented in pellet form. The MNC layer was recovered and washed with sterile PBS (500 g for 10 minutes). The cell pellet was completely resuspended in the remaining PBS and mixed with complete RPMI media at 10^7 cells/ mL.

2.9.10.2 Preparation of Yac-1 Target Cells

The Yac-1 target cells were labelled with CellTrace™ Violet (CTV) fluorescent dye (Life technologies Inc. Cat. No. C34557). Briefly, 1×10^6 Yac-1 cells were pelleted and mixed with 1 mL of pre-warmed sterile PBS. The cells were mixed with 1 µL of stock CTV and incubated for 20 minutes at room temperature with gentle agitation and protected from light. The unbound dye remained in solution was the quenched by adding 5 mL of pre-warmed RPMI media (10% FCS) and incubated for an extra 5 minutes at room temperature. Finally cells were pelleted and resuspended in RPMI at 10^5 cells/ mL.

2.9.10.3 Cell Trace Violet and PI staining Assay

A 96-well plate was prepared with 100 µL serial halving dilutions of splenic mononuclear cells with a range from 100:1 to 3:1 of effector cells: target cells (Figure 2.2). This was achieved by adding 200 µL of prepared MNCs in the 100:1 wells and 100 µL of RPMI media in the rest of the dilution wells. The MNCs (100 µL) was then transferred as a serial dilution through the relevant wells from 100:1 to 3:1. Finally, 100 µL of CTV-labelled Yac-1 cells were added to each well. A control well (0:1) was prepared with addition of 100 µL RPMI in 100 µL Yac-1 cell and no MNCs. All samples were loaded in triplicates.

Plates were incubated for 4 hours at 37 °C with 5% CO_2. PI solution (2.3.1.1) was diluted 1:25 with PBS and added to the wells right before analysis (25 µL). The amount of lysed Yac-1 cells representing NK cell cytotoxic activity was analysed using flow cytometry within two hours.

Flow cytometry data was analysed using Kaluza Software and the data was normalised by subtracting the percentage of spontaneous cell death observed in the wells with target cells only.

Note 1. The peak emission of CTV is 450 nm and was measured in the FL-1 channel.

Well	Dilutions	Serial Effector Cells Dilution	Target Cells
A	100:1	200 µL Splenic MNCs	100 µL Yac-1 cells
B	50:1	100 µL RPMI + 100 µL Splenic MNCs (previous well)	100 µL Yac-1 cells
C	25:1	100 µL RPMI + 100 µL splenic MNCs (previous well dilution)	100 µL Yac-1 cells
D	12:1	100 µL RPMI + 100 µL splenic MNCs (previous well dilution)	100 µL Yac-1 cells
E	6:1	100 µL RPMI + 100 µL splenic MNCs (previous well dilution)	100 µL Yac-1 cells
F	3:1	100 µL RPMI + 100 µL splenic MNCs (previous well dilution)	100 µL Yac-1 cells
G	0:1	100 µL RPMI	100 µL Yac-1 cells
H			

Figure 2.2. Cell trace violet and PI staining assay

2.10 Statistical Analysis

Data were analysed for significance using GraphPad Prism 5 Software. *In-vitro* experiments were performed in duplicate or triplicate. p values were determined using the Student's t-test accompanied by analysis of variance (ANOVA) as indicated in Figure legends. For *in vivo* experiments, a Kaplan-Meier survival curve was used to estimate the fraction of mice living for the certain amount of time after treatment. A p value of less than 0.05 was considered significant.

CHAPTER THREE

Cytotoxic Effects of Fucoidan on AML Cells

3.1 Introduction

Although chemotherapy is often effective in the treatment of AML, at least in the short term, the agents currently in use are associated with undesirable side effects and low survival rate (218). Concerns over toxicities from chemotherapeutic chemicals have generated interest in exploiting natural products for cancer treatment (219). Among those, fucoidan derived from brown seaweeds has gained significance in the treatment of cancer.

Different experimental studies have shown the tumour-suppressive activity of fucoidan in various human cancer cells, including gastric adenocarcinoma (220), prostate cancer (163), breast cancer (160), lung carcinoma (171) and colorectal cancer (167). Low toxicity and the *in vitro* cytotoxic effects of fucoidan on cancer cells make it a suitable choice for cancer prevention or treatment.

Despite evidence for the tumour inhibitory effect of fucoidan in different cancer types, there are only few studies which have examined the anti-proliferative property of fucoidan in haematological malignancies. Aisa *et al*. and Haneji *et al*. have reported that fucoidan induces apoptosis in human lymphoma HS-Sultan and adult T-cell leukaemia cell lines, respectively (155, 165). The first aim of this thesis chapter was to investigate whether anti-proliferative effects of fucoidan were observed in myeloid leukaemias *in vitro*.

Several studies have examined the mechanisms underlying the observed anti-tumour activities and provided some clues to the key pathways involved. Roles in apoptotic protein regulation (220), caspase activation (164) oxidative stress induction and mitochondrial dysfunction (167) have been reported. However the inconsistent and contradictory evidence to date has been challenging to interpret and the mechanisms of action of fucoidan remains unclear. The second aim of this chapter was to examine the mechanisms by which fucoidan affect cell growth in AML cells, and if the cytotoxic responses were observed in AML cells. To test this, a wide range of cellular and molecular pathways including cell proliferation, apoptosis, cell cycle and different signal transduction pathways were examined in AML cells after treatment with fucoidan.

3.2 Experimental Design

Fucoidan from different seaweed sources were obtained and their inhibitory effects were compared in AML cell growth.

The composition and characteristics of fucoidan from *F. vesiculosus* (Sigma-Aldrich Co.) and purified fucoidan from *U. pinnatifida* (Marinova Pty. Ltd.) were assessed and compared (see Section 2.8). Endotoxin contamination was a potential concern for the fucoidan preparation therefore whether endotoxin was present in the fucoidan preparation was determined (see Section 2.2).

AML cell lines including NB4, HL60 and K562 cells (see Section 2.1.1) were treated with Marinova Pty. Ltd. supplied purified fucoidan derived from two seaweed sources (*U. pinnatifida* and *F. vesiculosus*) where indicated with doses ranging from 10 to 2000 µg/mL. AML cell lines including NB4, HL60, K562 and KG-1a cells were then treated with fucoidan from *F. vesiculosus* (Sigma-Aldrich Co.) at various doses ranging from 10 up to 100 µg/mL. Cell proliferation was measured with WST-8 proliferation assay (see Section 2.4). Cell death and apoptosis were analysed by measuring DNA content, annexin V/PI and DNA fragmentation assays (see Sections 2.3, 2.5 and 2.6). To investigate the mechanisms by which fucoidan induced apoptosis, the expression of various proteins in cell cycle, apoptosis and signal transduction pathways were assessed using Western blotting (see Section 2.7).

3.3 Results

(This section has been substantially published in Atashrazm et al. 2015 (221))

3.3.1 Fucoidan Characteristics

3.3.1.1 Endotoxin Level Assay in Fucoidan

To evaluate the pyrogenicity and endotoxin contamination, the endotoxin level was measured in fucoidan. According to the "Guidelines on the Validation of the Limulus Amebocyte Lysate Test" defined by the FDA (12/87), the endotoxin level for human and animal parenteral drugs, biological products and medical devices must be ≤0.5 Endotoxin Units/ml (222). The endotoxin level of the fucoidan preparation was measured utilising the standard curve generated from *E. coli* endotoxin standards. Less than 0.1 EU/mL endotoxin was detected in fucoidan preparation, which is considered as non-pyrogenic.

3.3.1.2 Analysis of the Two Fucoidan Preparations

The characteristics of two sources of fucoidan were evaluated; fucoidan from *F. vesiculosus* supplied by Sigma-Aldrich Co. and purified fucoidan from *U. pinnatifida* supplied by Marinova Pty. Ltd. The experiments for characterisation of the fucoidan constituents were performed by Samuel S Karpiniec at Marinova Pty. Ltd. The results for the compositional analysis of the fucoidans from Sigma-Aldrich Co. and Marinova Pty. Ltd. are shown in Table 3.1. As indicated, fucoidan from *F. vesiculosus* (Sigma-Aldrich Co.) had lower molecular weight and less fucose content compared to the Marinova Pty. Ltd. supplied fucoidan from *U. pinnatifida*. The amount of other monosaccharaides in addition to sulphate content were similar in fucoidan from both sources. The amount of uronic acid in fucoidan from Sigma-Aldrich Co. was 4 fold higher than that of fucoidan from Marinova Pty. Ltd. The purity of the fucoidan from Sigma-Aldrich Co. was near 80% compared to 98% in purified fucoidan (Marinova Pty. Ltd.). In addition, 3.9% polyphenol was found in the Sigma-Aldrich Co. supplied fucoidan structure.

Table 3.1. Composition and monosaccharide constituents of fucoidan from different sources

		Fucoidan from *F. vesiculosus* (Sigma-Aldrich Co.)	Fucoidan from *U. pinnatifida* (Marinova Pty. Ltd.)
Molecular Weight (kDa)		20.7	61.7
Fucoidan (%)		79.3	98.0
Total Carbohydrate (%)		55.9	67.5
Uronic Acid (%)		5.9	1.4
Polyphenol (%)		3.9	-
Cations (%)		6.4	11.9
Sulphate (%)		26.9	26.6
Monosaccharide Composition (%)	**Fucose**	38.2	53.3
	Xylose	2.7	2.3
	Mannose	0.4	0.8
	Galactose	3.5	3.1
	Glucose	0.1	0.0

3.3.2 Purified Fucoidans from *U. pinnatifida* and *F. vesiculosus* (Marinova Pty. Ltd.) Did Not Suppress the Growth of AML Cells

To determine whether fucoidan inhibits the growth of AML cells through either cell cycle arrest or cell death, cell cycle progression was assessed in cells treated with purified fucoidan (Marinova Pty. Ltd.). This was performed by DNA quantitation whereby DNA is labelled and the amount of measured dye quantitated (84). Figure 3.1 (left) displays the typical DNA content histogram in a healthy cell population. As indicated, the G0/G1 phase cells have the least amount of staining intensity as they have the least amount of genetic material (only one copy of DNA). G2/M phase cells, in contrast, have the highest amount of staining intensity as they contain the highest amount of DNA (two copies of chromosomes). Since the S phase cells are synthetising DNA, they have different amounts of DNA varying from one copy to two copies.

The cell cycle histogram not only gives us information about the status of the living cells, but also detects the dead cells. As DNA is degraded in the late stages of the death process, a large number of small DNA fragments accumulate in the cells, which can then leach out. Since these dying cells have a lower DNA content, they can be observed in the cell cycle histogram as a hypo-diploid or sub G0/G1 peak (223). Figure 3.1 compares the cell cycle histogram between a normal cell population and a dead cell population.

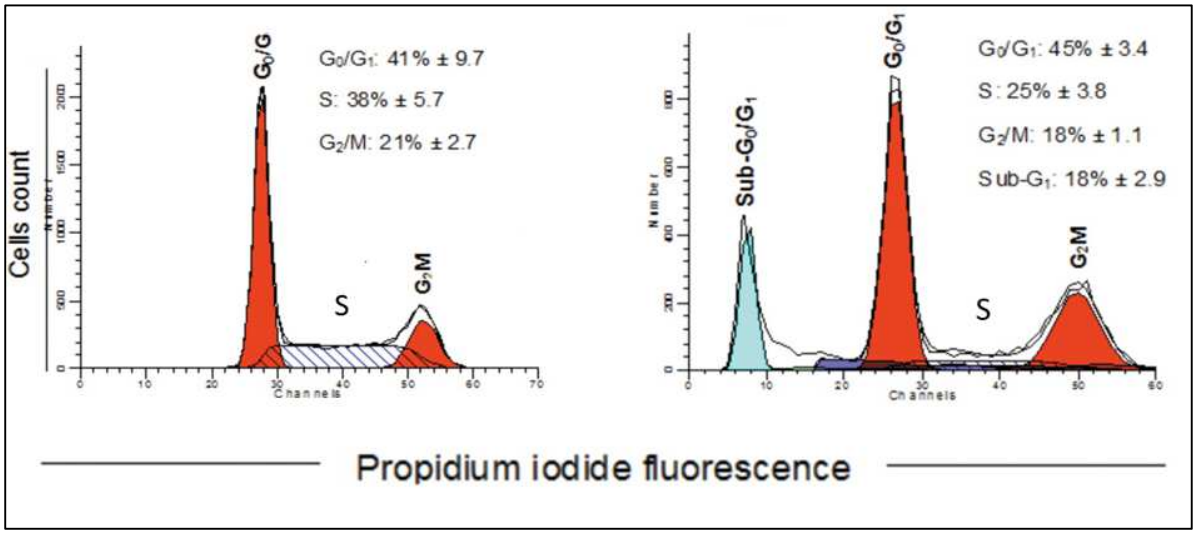


Figure 3.1. Cell cycle histogram in healthy (left) and apoptotic (right) population (224)

Phorbol 12-myristate 13-acetate (PMA) was used as a positive control, to ensure that cells were responding to chemical agents as expected and to assess efficacy of the assay methodology. PMA is a diester of phorbol which induces G0/G1 cell cycle arrest in a variety of cell types. The leukaemic cells were treated with 20 ng/mL PMA for 48 hours and DNA content was measured using PI staining. As shown in Figure 3.2, PMA inhibited cell proliferation in K562, NB4 and HL60 cells, which was indicated by G0/G1 arrest in all three cell lines.

To investigate the growth inhibitory effect of fucoidan, acute myeloid leukaemia cell lines HL60, NB4 and K562 were treated with the two different purified fucoidans from Marinova Pty. Ltd. as indicated with doses up to 150 µg/mL. After 48 hours, the DNA content was analysed using PI staining. Neither fucoidan preparations influenced the proportion of cells present in each of the cell cycle phases as measured by flow cytometry at any of the given doses (Figures 3.3, 3.4, 3.5) in the experimental time frame. A similar result was observed in each of the cell lines examined.

Furthermore, very high doses of the two purified fucoidans from Marinova Pty. Ltd (for up to 2 mg/mL) were used and no changes were observed in any phases of the cell cycle for either fucoidan preparations. Figures 3.6 and 3.7 display DNA content in HL60 cells after treatment with very high dose of the two purified fucoidans from Marinova Pty. Ltd.

DNA fragmentation was also assessed in fucoidan-treated cells at a low (150 µg/mL) and a very high dose (2000 µg/mL). In agreement with the DNA content results, the purified fucoidan (Marinova Pty. Ltd.) did not induce DNA fragmentation in the treated cell lines (Figure 3.8).

In summary, purified fucoidans from two sources of *U. pinnatifida* and *F. vesiculosus* supplied by Marinova Pty. Ltd. did not affect cell proliferation, DNA content and DNA fragmentation in different AML cell lines as indicated. Due to inconsistency with literature, this was further examined by analysing the anti-proliferative activity of a commercially available fucoidan extract, which is the most common source of experimental fucoidan used in literature.

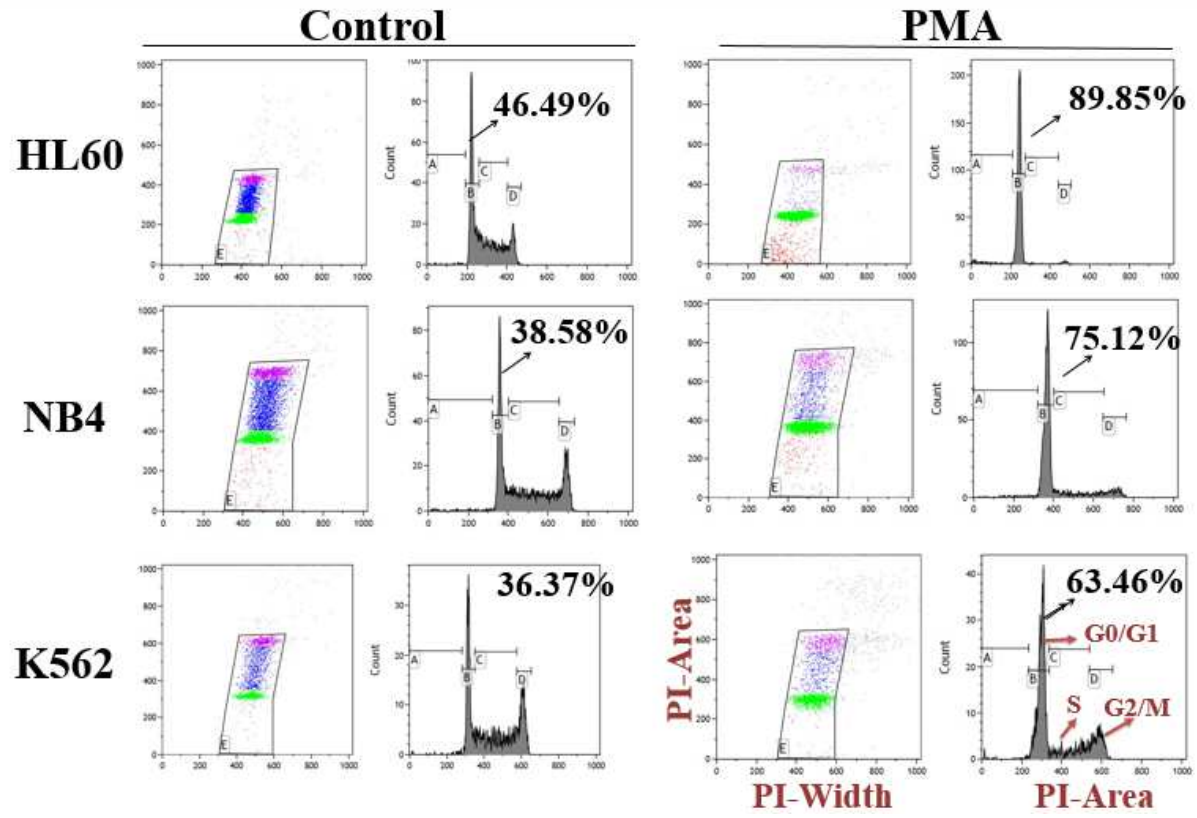


Figure 3.2. DNA content analysis in AML cells treated with PMA. HL60, NB4 and K562 cell lines were treated with 20 ng/mL PMA, and cell cycle phase distribution was measured using PI staining and quantitated by flow cytometry.

Green population: G0/G1 phase cells, **Blue population**: S phase cells, **Purple population**: G2/M phase cells

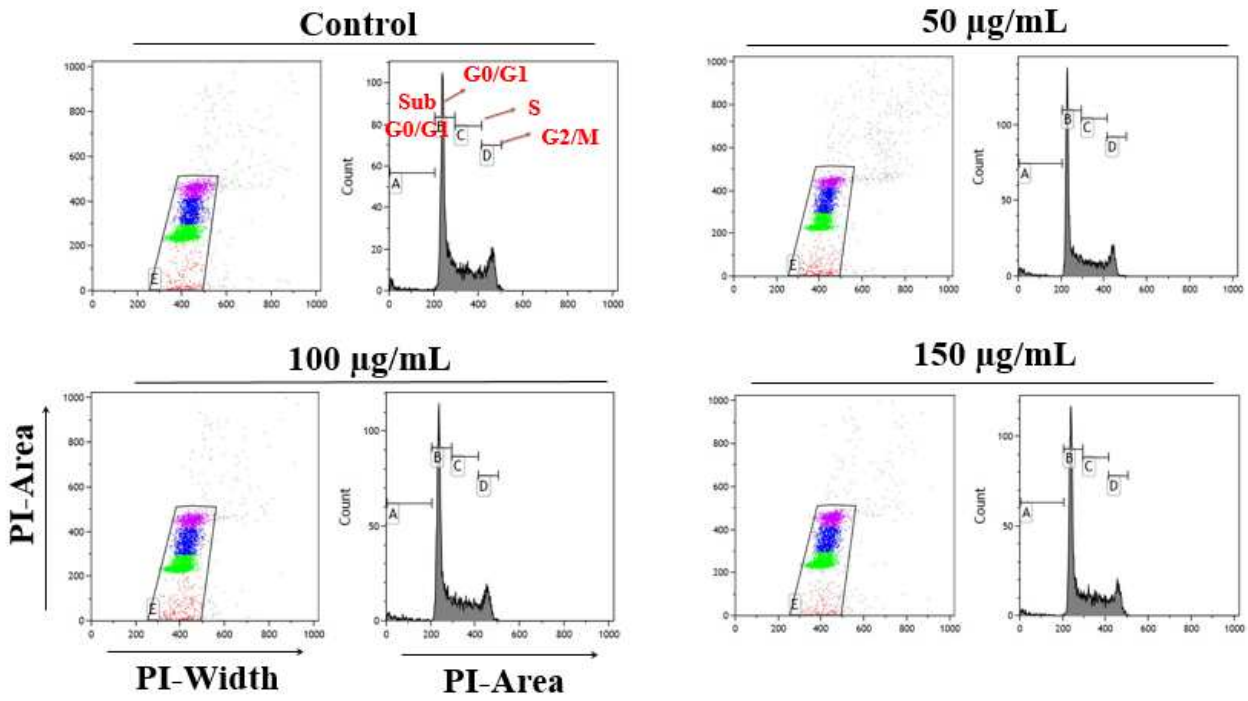


Figure 3.3. DNA content analysis in HL60 cells. HL60 cells were treated with increasing doses (from 50 µg/mL to 150 µg/mL) of fucoidan from *U. pinnatifida* (Marinova Pty. Ltd.), and cell cycle phase distribution was measured using PI staining and quantitated by flow cytometry.

Red population: Sub-G0/G1 phase cells, **Green population**: G0/G1 phase cells, **Blue population**: S phase cells, **Purple population**: G2/M phase cells

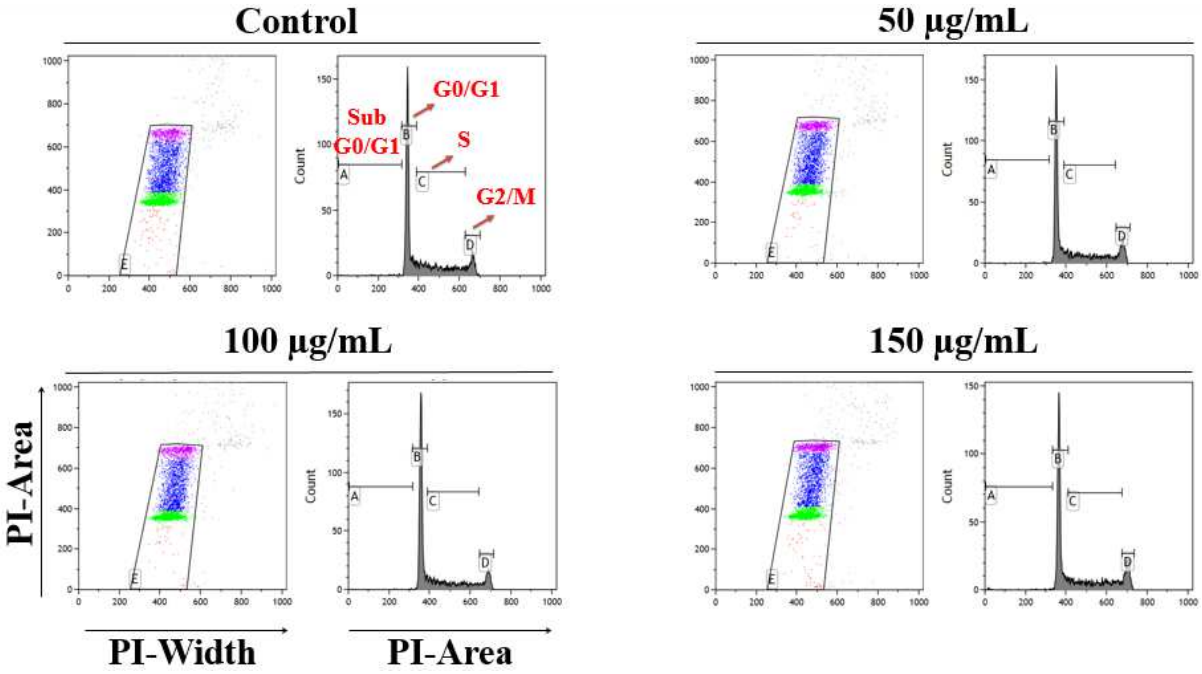


Figure 3.4. DNA content analysis in NB4 cells. NB4 cells were treated with increasing doses (from 50 µg/mL to 150 µg/mL) of fucoidan from *U. pinnatifida* (Marinova Pty. Ltd.), and cell cycle phase distribution was measured using PI staining and quantitated by flow cytometry.

Red population: Sub-G0/G1 phase cells, **Green population**: G0/G1 phase cells, **Blue population**: S phase cells, **Purple population**: G2/M phase cells

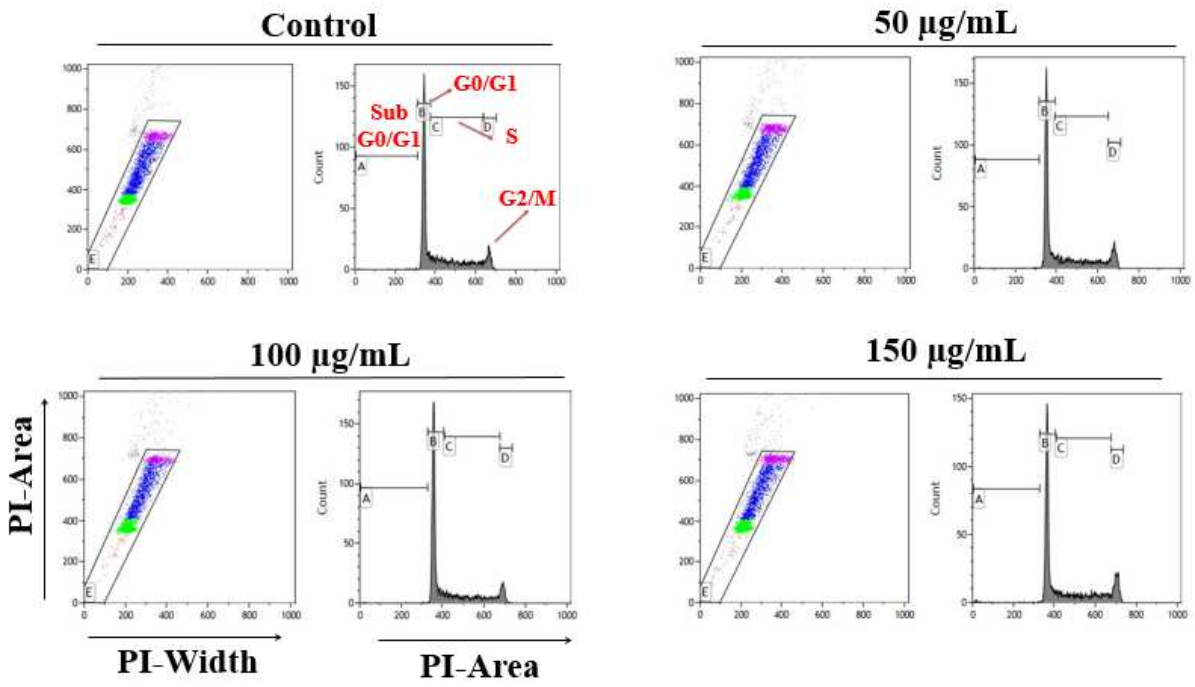


Figure 3.5. DNA content analysis in K562 cells. K562 cells were treated with increasing doses (from 50 µg/mL to 150 µg/mL) of fucoidan from *U. pinnatifida* (Marinova Pty. Ltd.), and cell cycle phase distribution was measured using PI staining and quantitated by flow cytometry.

Red population: Sub-G0/G1 phase cells, **Green population**: G0/G1 phase cells, **Blue population**: S phase cells, **Purple population**: G2/M phase cells

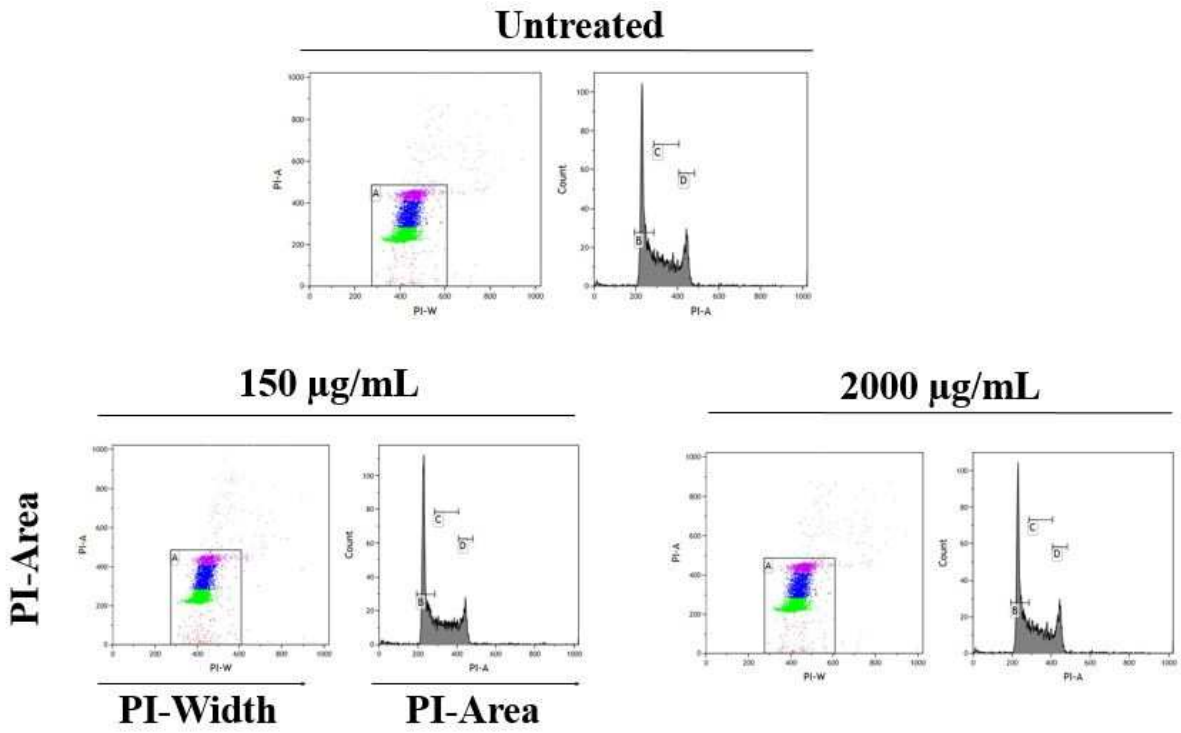


Figure 3.6. DNA content analysis in HL60 cells treated with high dose fucoidan. HL60 cells were treated with low (150 µg/mL) and high (2000 µg/mL) doses of fucoidan from *U. pinnatifida* (Marinova Pty. Ltd), and cell cycle phase distribution was measured using PI staining and quantitated by flow cytometry.

Red population: Sub-G0/G1 phase cells, **Green population**: G0/G1 phase cells,
Blue population: S phase cells, **Purple population**: G2/M phase cells

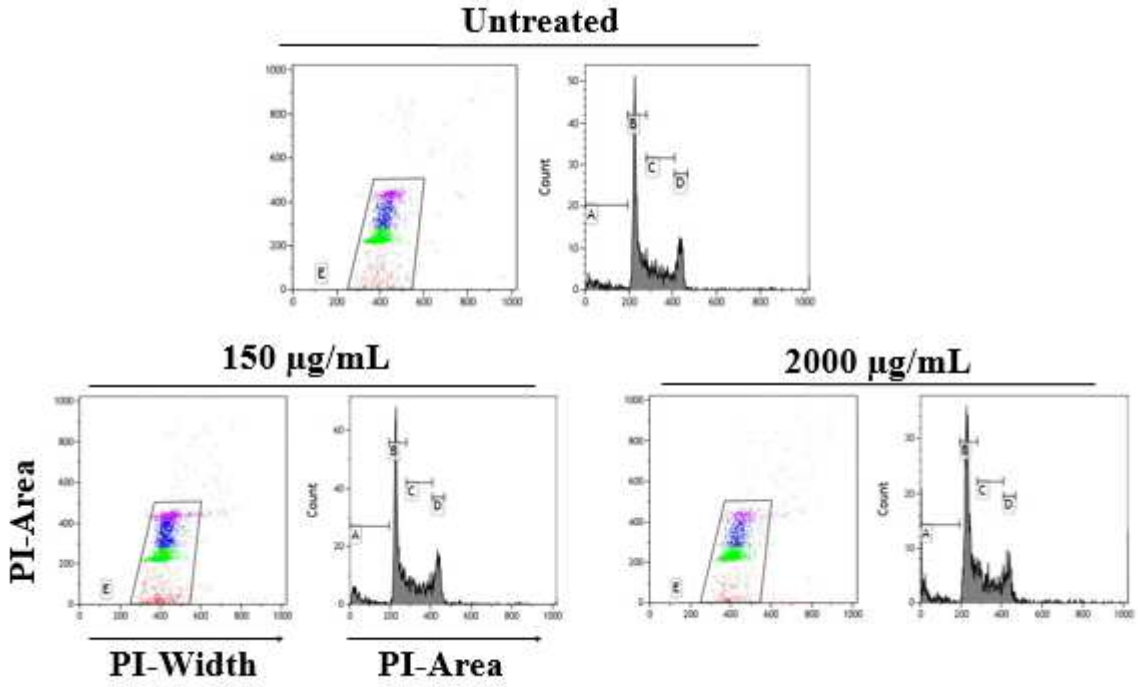


Figure 3.7. DNA content analysis in HL60 cells treated with high dose fucoidan. HL60 cells were treated with low (150 µg/mL) and high (2000 µg/mL) doses of fucoidan from *F. vesiculosus* (Marinova Pty. Ltd), and cell cycle phase distribution was measured using PI staining and quantitated by flow cytometry.

Red population: Sub-G0/G1 phase cells, **Green population**: G0/G1 phase cells, **Blue population**: S phase cells, **Purple population**: G2/M phase cells

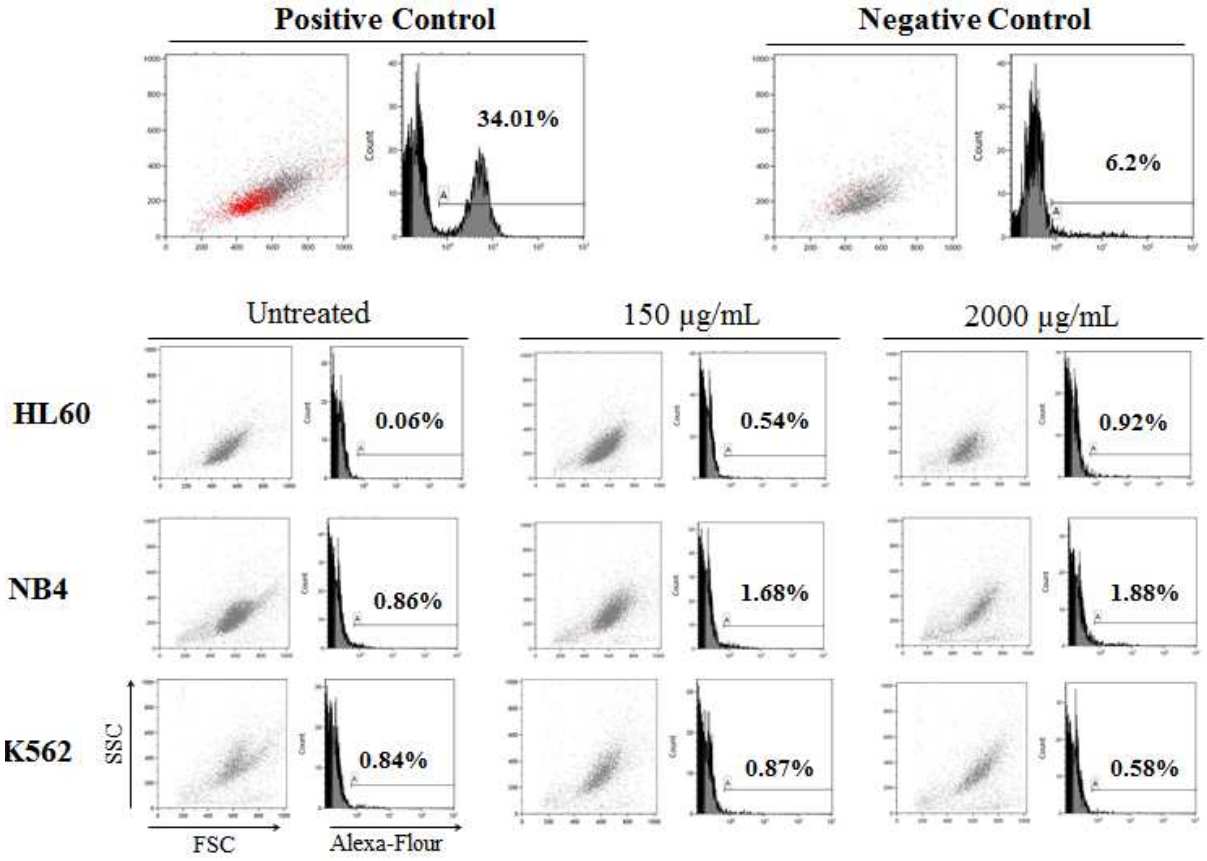


Figure 3.8. DNA fragmentation assay in AML cells. HL60, NB4 and K562 cell lines were treated with low (150 µg/mL) and high (2000 µg/mL) doses of fucoidan from *U. pinnatifida*, and DNA fragmentation was assessed using TUNEL assay.

Red population: Dead cells with DNA fragmentation, **Black population**: Live cells

3.3.3 Cytotoxicity Activity of Fucoidan (Sigma-Aldrich Co.) in AML Cells

Fucoidan from *F. vesiculosus* was obtained from Sigma-Aldrich Co. and its cytotoxicity and antiproliferative activities were examined in different types of AML.

3.3.3.1 Fucoidan Inhibited Cell Proliferation in NB4, HL60 and K562 Cell Lines

To investigate the proliferation inhibitory effects of this fucoidan preparation on AML cells, cell lines were treated with increasing doses of fucoidan and after 48 hours cell proliferation was measured with the WST-8 proliferation assay (Figure 3.9). The results indicated that fucoidan differentially inhibited cell growth in selected leukaemia cell sub-types; it inhibited the proliferation of acute promyelocytic leukaemia NB4 and HL60 cell lines in a dose dependent manner with the level of cell proliferation decreased to less than 10% at concentrations of 25 and 50 µg/mL in NB4 and HL60 cells, respectively. To a lesser extent, fucoidan reduced the proliferation of K562 cells and the amount of cell proliferation remained more than 70% at a dose of 100 µg/mL. In contrast, fucoidan had almost no effect on KG-1a cell proliferation.

3.3.3.2 Fucoidan Increased Sub-G0/G1 Fraction in NB4 and HL60 Cells.

To investigate whether the reduced proliferation in NB4 and HL60 cells was due to cell cycle arrest or increased cell death, cells were treated with fucoidan and DNA content was evaluated for up to 48 hours using PI staining. The percentage of sub-G0/G1 fraction representing the dead cell population significantly increased in both HL60 and NB4 cells in a time dependent manner (Figure 3.10 A, B). Table 3.2 shows the percentage of cells in each phase of the cell cycle as measured by PI staining using flow cytometry.

Furthermore, the effects of fucoidan on cell cycle was determined in K562 and KG-1a cell lines. A cell cycle arrest in G0/G1 accompanied by a decrease in S and M phases was observed in fucoidan-treated K562 cells (Figure 3.10 C); approximately 60% of the cells treated with fucoidan were in the non-proliferative fraction compared to around 40% in untreated cells (Table 3.2).

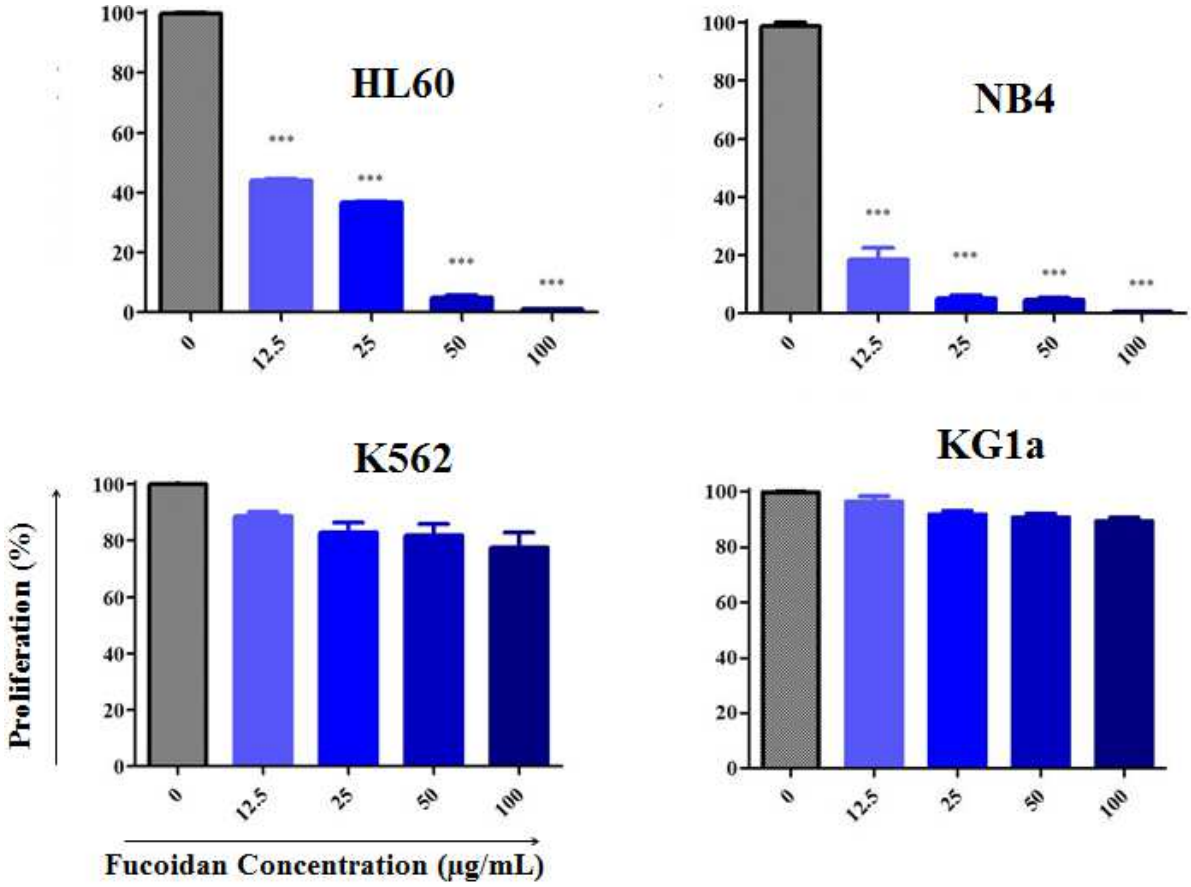


Figure 3.9. Inhibitory effect of fucoidan on cell proliferation. Different leukaemia cell lines were treated with increasing doses of fucoidan (Sigma-Aldrich Co.) (12.5 µg/mL to 100 µg/mL) and measured for proliferation with the WST-8 assay. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test (***: $p \leq 0.001$, **: $p \leq 0.01$).

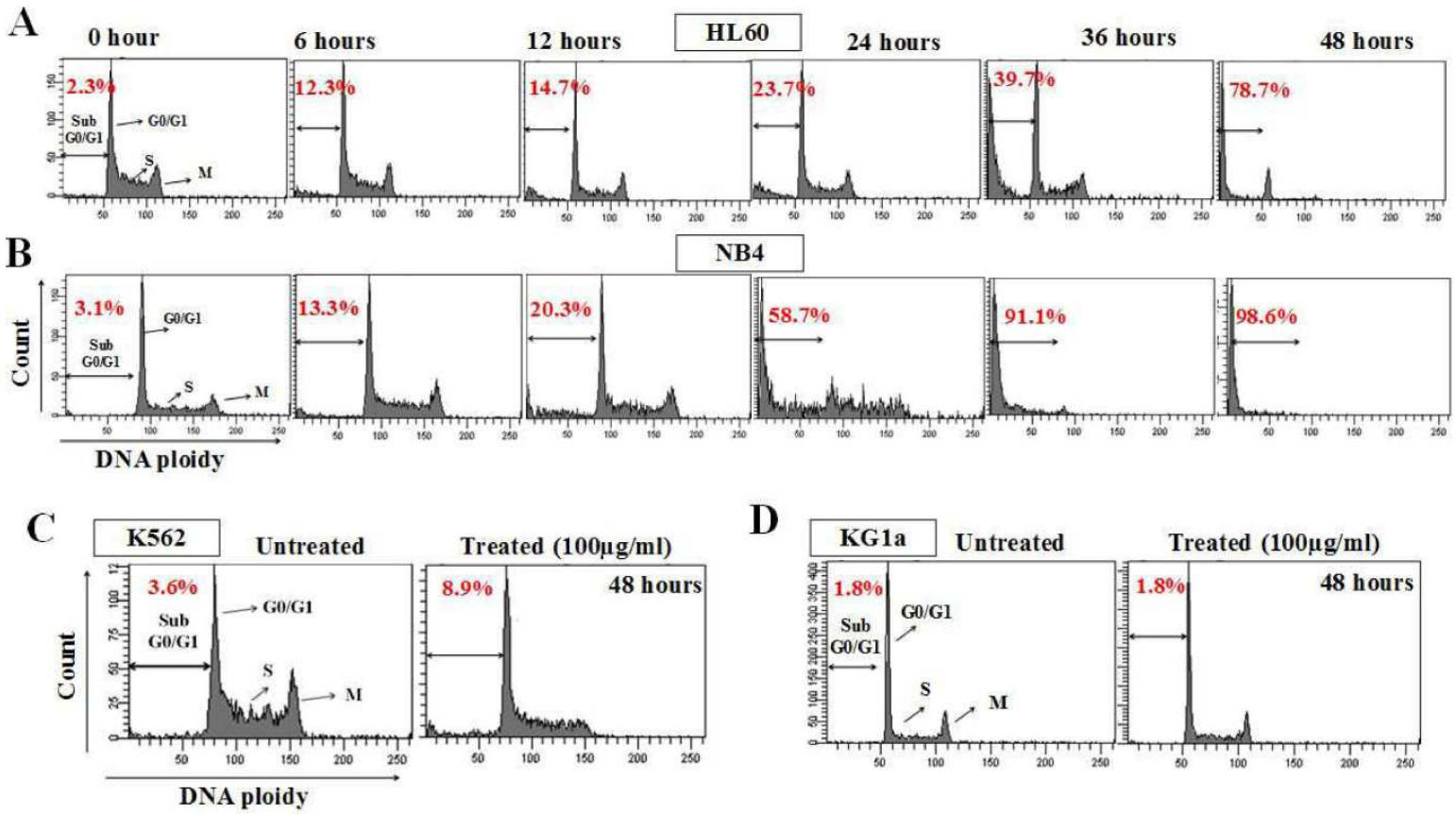


Figure 3.10. Representative DNA content analysis. A, B) HL60 and NB4 cells were treated with 100 µg/mL fucoidan and DNA content was analysed for up to 48 hours. The sub-G0/G1dead cell fraction increased in a time-dependent manner ($p \leq 0.001$). **C, D)** K562 and KG-1a cells were respectively treated with 100 µg/mL fucoidan for 48 hours and DNA content was analysed.

Table 3.2. Cell cycle phases distribution. DNA content was assessed in different AML cell lines after treatment with 100 µg/mL fucoidan for 48 hours. Data represents Mean ± SEM of three independent experiments. Statistical significance was determined by two-way ANOVA, followed by Bonferroni post-test (***: p≤0.001).

Cell line	Fucoidan	Sub G0/G1 (%)	G0/G1 (%)	S (%)	M (%)
HL60	Untreated	**2.5** ± 1.26	**48.4** ± 1.26	**29.0** ± 1.04	**18.8** ± 3.27
	Treated	**78.7** ± 2.45***	**8.3** ± 1.35	**7.0** ± 2.95	**6.3** ± 1.81
NB4	Untreated	**5.9** ± 2.45	**43.3** ± 2.46	**32.3** ± 1.72	**16.2** ± 1.34
	Treated	**97.5** ± 3.004***	**1.5** ± 1.98	**0.6** ± 0.68	**0.1** ± 0.11
K562	Untreated	**3.3** ± 0.20	**42.6** ± 4.66	**30.4** ± 3.05	**21.5** ± 1.79
	Treated	**9.0** ± 0.80	**58.3** ± 2.482	**24.4** ± 0.92	**7.7** ± 1.69
KG-1a	Untreated	**1.6** ± 0.41	**61.1** ± 1.08	**15.9** ± 0.66	**20.7** ± 1.85
	Treated	**1.3** ± 0.40	**60.3** ± 1.19	**16.0** ± 1.19	**21.6** ± 1.14

Unlike the HL60, NB4 and K562 cell lines, the undifferentiated AML cell line KG-1a was resistant to the cell cycle modulatory effects of fucoidan (Figure 3.10 D). This was consistent with the proliferation assay results as fucoidan neither induced cell cycle arrest nor sub-G0/G1 dead cell population in KG-1a cells (Table 3.2).

Taken together, the results indicate that fucoidan reduced proliferation of HL60 and NB4 cell lines at the sub-G0/G1 stage and reduced proliferation of K562 cells at the G0/G1 stage, but did not inhibit the KG-1a cell growth.

3.3.3.3 Fucoidan Induced Apoptosis in NB4 and HL60 Cells.

The observed reduced cell proliferation in NB4, HL60 and K562 cells and increased sub-G0/G1 dead cell population in HL60 and NB4 cells were further examined. The annexin V/PI apoptosis assay and DNA fragmentation TUNEL assays were employed to measure apoptosis and evaluate mechanisms of cell death induced by fucoidan.

The annexin V/PI apoptosis assay permits the distinction between apoptosis and necrosis to be observed. During the earliest stages of apoptosis there is a loss of asymmetry of the plasma membrane, which results in the translocation of the phosphatidylserine (PS) from the inner side of the cell membrane to the surface. The exposed PS can then bound to annexin V. In the very earliest stages of apoptosis the membrane remains impermeable to the dye PI. Upon the loss of membrane integrity at the late stages of apoptosis however PI passes through the cell. Necrotic cells are also permeable to PI but its binding to annexin V is relatively low and this allows discrimination of early and late apoptotic cells from necrotic cells (225) (Figure 3.11).

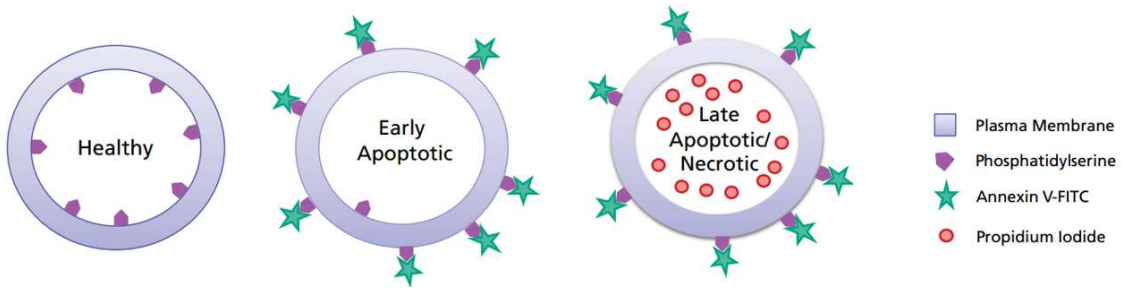


Figure 3.11. Healthy and apoptotic cells with markers for detection of apoptosis (226)

As shown in Figures 3.12 and 3.13, the annexin V/PI apoptosis assay revealed that fucoidan significantly increased apoptosis in HL60 ($p \leq 0.01$) and NB4 cells ($p \leq 0.001$). Consistent with the proliferation and DNA content assay results, fucoidan failed to induce apoptosis in K562 and KG-1a cell lines (Figure 3.12).

DNA fragmentation, which is a feature of late apoptosis, was examined in fucoidan-treated cells by TUNEL assay in triplicate and the mean values were analysed (Figure 3.14). DNA fragmentation significantly increased in HL60 and NB4 cells in a dose dependent manner. Compared to untreated cells, the percentage of cells containing fragmented DNA increased from less than 1% to 88.9% in NB4 cells and to 54.8% in HL60 cells treated with 100 µg/mL fucoidan ($p \leq 0.01$, $p \leq 0.001$). In contrast, the DNA fragmentation amount remained unchanged in K562 and KG-1a cells treated with up to 100 µg/mL fucoidan (less than 1%).

Taken together, these results indicate that apoptosis induction by fucoidan within tumour cells is cell-type specific and further that promyelocytic cells were more sensitive to fucoidan's action. In the next step, the underpinning mechanisms of the fucoidan's activity in induction of apoptosis was investigated.

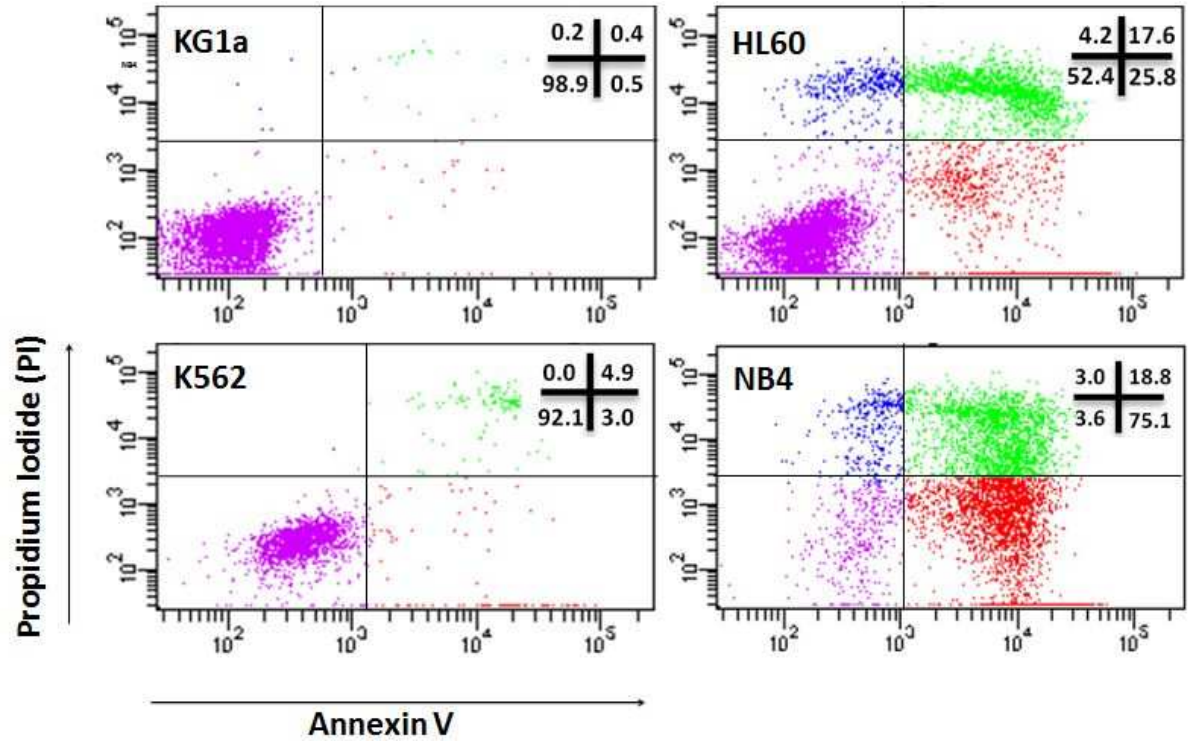


Figure 3.12. Annexin V/PI apoptosis assay in AML cell lines trated with fucoidan. Apoptosis was evaluated using annexin V/PI assay. Cell lines were treated with 100 µg/mL fucoidan. Results represent one of three independent experiments.

*: **Purple population**: Annexin$^-$/PI$^-$ (live cells), **Blue population**: Annexin$^-$/PI$^+$ (necrotic cells), **Red population**: Annexin$^+$/PI$^-$ (early apoptotic cells), **Green population**: Annexin$^+$/PI$^+$ (late apoptotic cells),

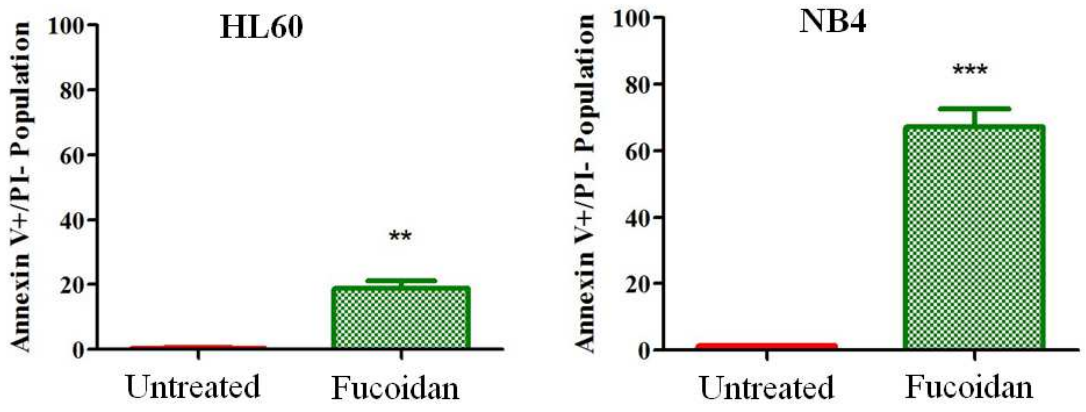


Figure 3.13. Early apoptosis detection. HL60 and NB4 cells were treated with 100 µg/mL fucoidan for 24 hours. Apoptosis was assessed using Annexin V/PI assay. Annexin V positive/PI negative populations representing early apoptosis were calculated. Each column represents the Mean ± SEM (n=3) values for annexin V positive/PI negative population. Statistical significance was determined by one-tailed student t test. (***: $p \leq 0.001$, **: $p \leq 0.01$).

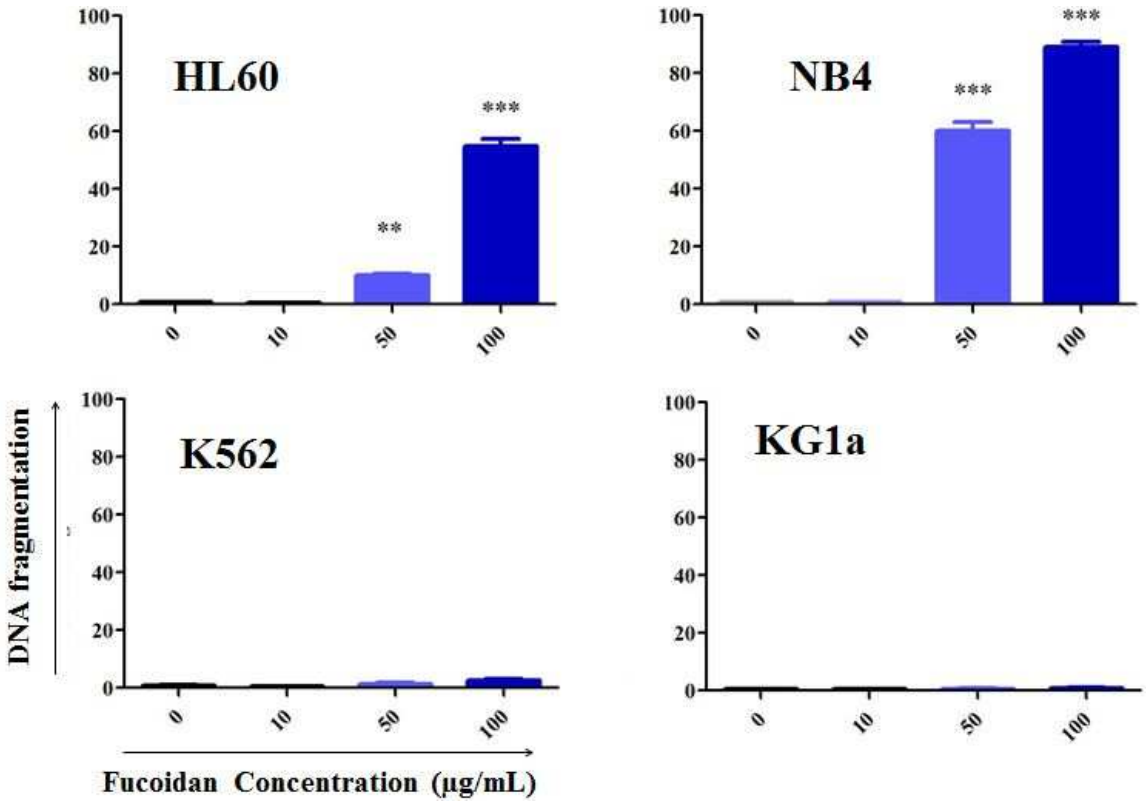


Figure 3.14. DNA fragmentation TUNEL assay. HL60, NB4, K562 and KG-1a cells were treated with increaing doses of fucoidan (from 10-100 µg/mL) and DNA fragmentation was measured using the TUNEL assay. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test (***: $p \leq 0.001$, **: $p \leq 0.01$).

3.3.3.4 Fucoidan-Induced Apoptosis was Caspase Dependent

As NB4 cells showed the highest sensitivity to fucoidan, the further experiments were conducted on NB4 cells. To determine the mechanisms by which fucoidan induces apoptosis, the activation of caspase 3 as well as the cleavage of poly ADP ribose polymerase (PARP), a downstream target of activated caspase 3 in isolated nuclei, were examined using Western blotting. Since fucoidan caused near 100% NB4 cells death within 48 hours, the shorter incubation time of 24 hours was selected for this assay in order to obtain sufficient amount of protein. Fucoidan decreased the level of pro-caspase 3 and increased the cleaved activated caspase 3 in treated cells in a dose dependent manner correlated with the detection of cleaved PARP (Figure 3.15).

In order to examine the importance of caspase participation in apoptosis-induced by fucoidan, cell proliferation and annexin V apoptosis assays were performed in the presence of the caspase inhibitor Z-VAD-fmk in NB4 cells. In the WST-8 assay, the caspase inhibitor Z-VAD-fmk was protective and partially ameliorated the anti-proliferative effect of fucoidan ($p \leq 0.001$) (Figure 3.16 A). In the annexin V/PI apoptosis assay, the inhibitory effect of fucoidan was completely annulled in the presence of Z-VAD-fmk ($p \leq 0.001$) (Figure 3.16 B), indicating that caspase activation is required for fucoidan activity.

The expression of various apoptosis, cell cycle and signalling pathway related molecules were then examined. For these experiments, a dose of 50 µg/mL and the incubation time of 24 hours were selected in order to obtain sufficient amount of protein.

To evaluate different apoptosis pathways, the expression of death receptors DR5 and Fas, pro-apoptotic molecule Bax and anti-apoptotic molecule Bcl-xl in addition to the activation of caspases 8 and 9 were examined. There was an increase in the amount of cleaved activated caspase 8, which correlated to DR5 activation (Figure 3.17 A). No change in Fas expression was observed. Caspase 9, the key caspase in the intrinsic pathway, was also activated by fucoidan. Fucoidan increased expression of the pro-apoptotic molecule Bax but did not affect expression of the anti-apoptotic molecule Bcl-xl (Figure 3.17 B).

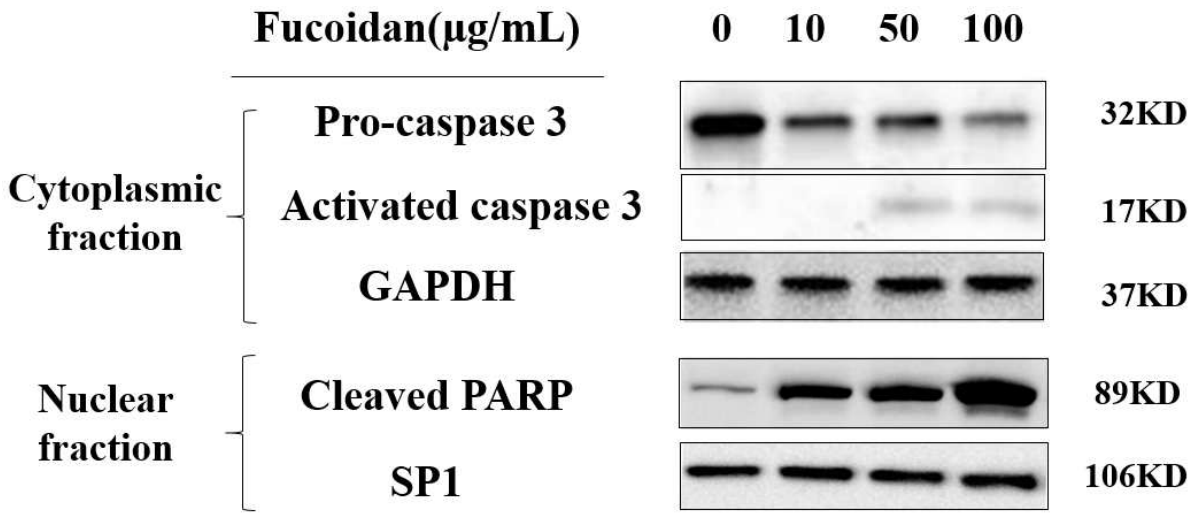


Figure 3.15. Analysis of caspase 3 activation and PARP cleavage in fucoidan-induced apoptosis by Western blot. NB4 cells were treated with various concentrations of fucoidan for 24 hours and cytoplasmic and nuclear fractions were isolated. GAPDH and SP1 were used as cytoplasmic and nuclear loading controls, respectively.

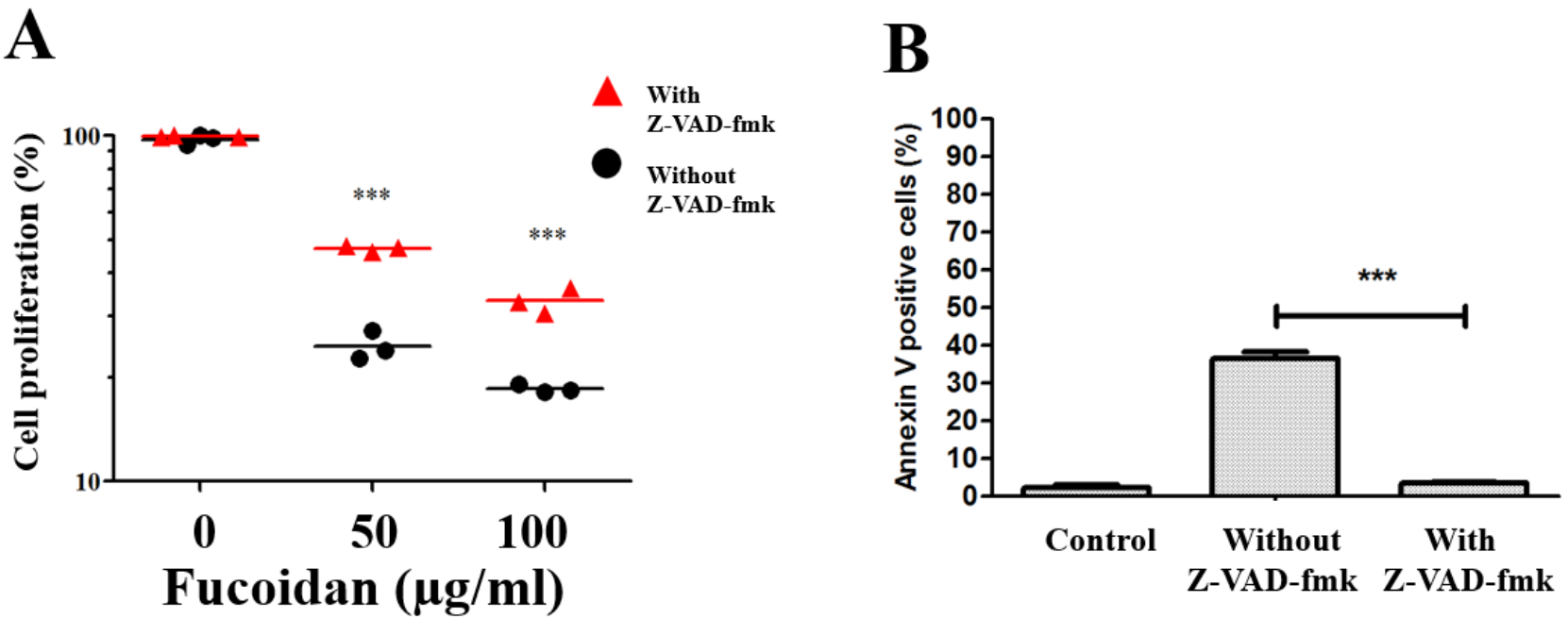


Figure 3.16. Effects of caspase inhibitor on fucoidan induced apoptosis. NB4 cells were pre-treated with Caspase inhibitor Z-Vad-fmk (40µM) for 1 hour followed by treatment with fucoidan for 24 hours. **A)** Cell proliferation was assesed using WST-8 proliferaton assay. **B)** Apoptosis was analysed using Annexin V/PI apoptosis assay. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by two-way ANOVA, followed by Bonferroni post-test (***: $p \leq 0.001$).

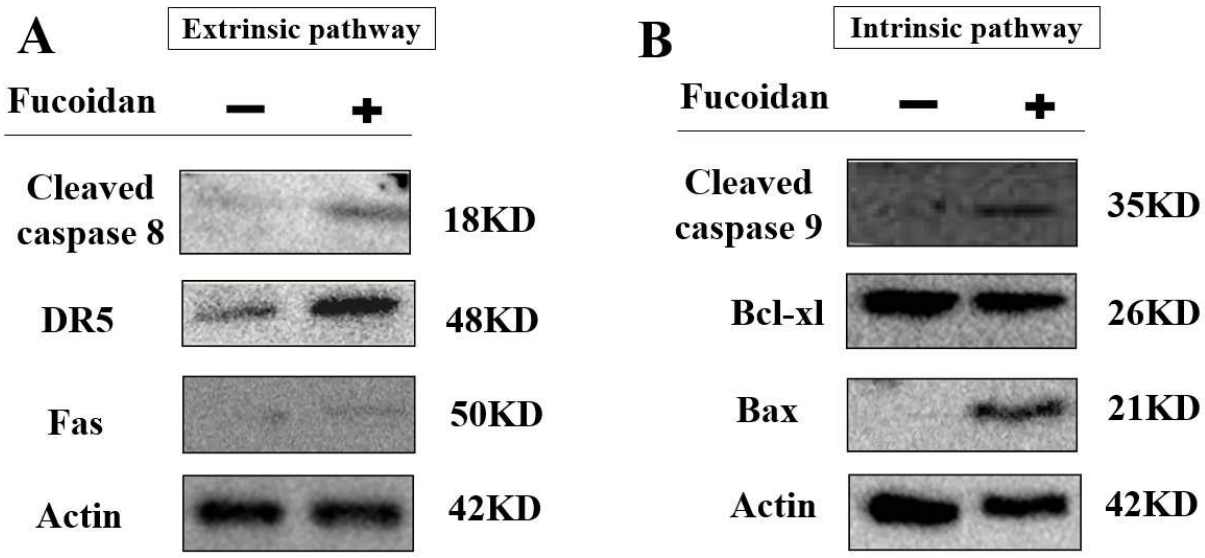


Figure 3.17. Analysis of the expression of apoptosis related proteins in A) extrinsic pathway B) intrinsic pathway by Western blot. NB4 cells were treated with 50 µg/mL fucoidan for 24 hours and cytoplasmic fraction was isolated. Protein expression levels of cleaved caspase 8, DR5, Fas, cleaved caspase 9, Bcl-xl and Bax were analysed using specific antibodies. The cytoplasmic protein β-actin was used as loading control.

3.3.3.5 Fucoidan Changed the expression of Cell Cycle Proteins

Since fucoidan altered the distribution of cell cycle phases in NB4 cell lines, the expression of cell cycle regulatory proteins P21/WAF1/CIP1 and cyclin D1 were examined using Western blotting. The cyclin dependent kinase inhibitor P21/WAF1/CIP1 is a negative cell-cycle-regulatory protein. Treatment with 50 µg/mL fucoidan for 24 hours induced a marked increase in P21/WAF1/CIP1 expression in NB4 cells. No changes were seen in cyclin D1 expression (Figure 3.18).

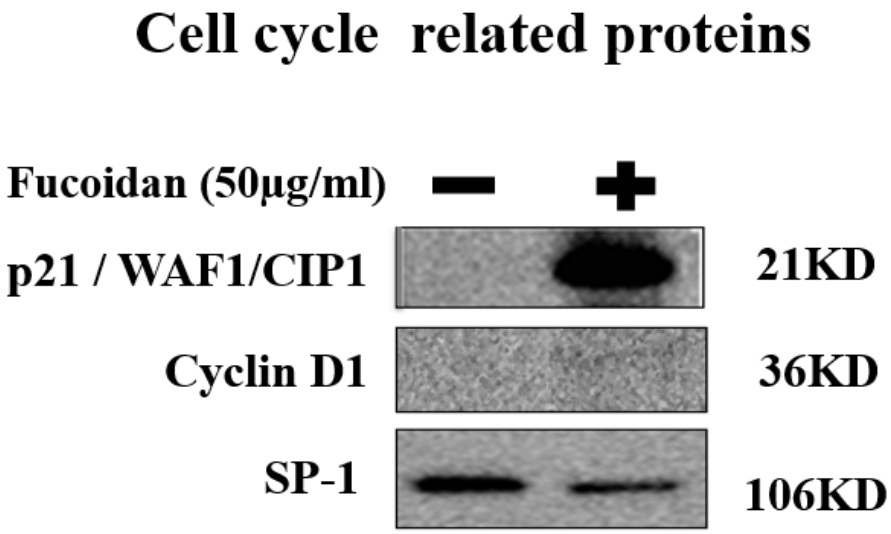


Figure 3.18. Analysis of the expression of cell cycle related proteins by Western blot. NB4 cells were treated with 50 µg/mL fucoidan for 24 hours and nuclear fraction was isolated. Protein expression and P21/WAF1/CIP1 and cyclin D1 were analysed using specific antibodies. The nuclear protein SP-1 was used as loading control.

3.3.3.6 Fucoidan Deactivated ERK1/2 and Decreased Phosphorylation of AKT on Thr308 Residue.

MAPK and PI3k/AKT signal transduction pathways play major roles in cell proliferation and apoptosis, hence both expression and activation levels of two main kinases were examined in these pathways; ERK in the MAPK pathway and AKT in the P13K pathway. Whilst total protein expression level of these two enzymes was not altered with fucoidan treatment of NB4 cells, the phosphorylation of both AKT and ERK1/2 enzymes was clearly down-regulated (Figure 3.19), AKT is a serine/threonine-specific protein kinase and has two different phosphorylation sites; Thr308 and Ser473. By examining the phosphorylation status of AKT at both sites it was found that fucoidan did not alter the phosphorylation of AKT on the Ser473 site in APL cells, but it down-regulated the phosphorylation of AKT on the Thr308 residue as indicated.

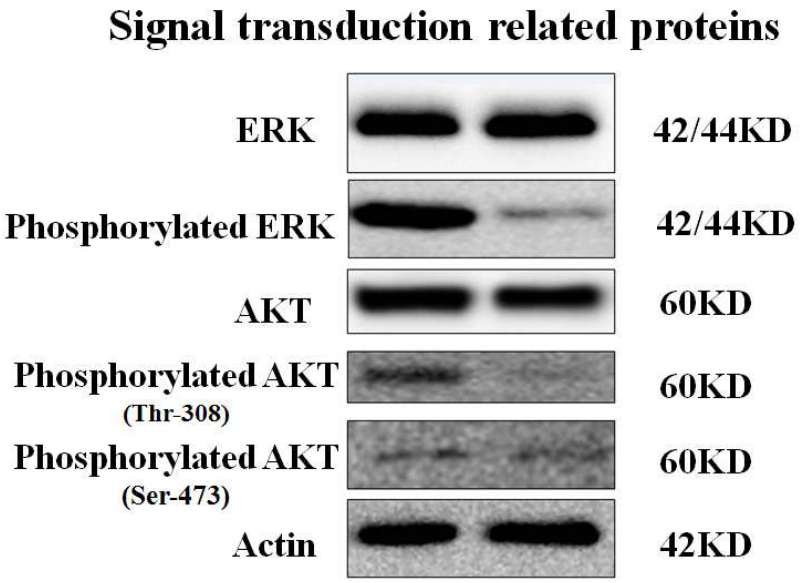


Figure 3.19. Analysis of the signal transduction pathways involvement in fucoidan-mediated apoptosis by Western blot. NB4 cells were treated with 50 µg/mL fucoidan for 24 hours. Protein expression and phosphorylation levels of ERK and AKT were analysed using specific antibodies. The cytoplasmic protein β-actin was used as loading control.

3.3.3.7 Cellular Signalling and Fucoidan

To investigate whether fucoidan enters into the cells or functions as an extracellular stimulus, NB4 cells were pre-treated with various concentrations of monensin, an endocytosis blocker, for 2 hours following treatment with fucoidan for 24 hours. Cell proliferation was analysed using WST-8 proliferation assay. It was observed that the endocytosis blockage did not prevent the NB4 cell growth inhibition induced by fucoidan (Figure 3.20). However, since the endocytosis blocker itself had growth-inhibitory effect and reduced the proliferation of NB4 cells, the obtained combined-(fucoidan+blocker)-treated-cells data were normalised according to blocker only-treated cells. After normalization, no significant difference was observed between fucoidan-treated cells with or without pre-treatment with endocytosis blocker (Figure 3.21). Pre-treatment with endocytosis blocker did not restore the reduced cell proliferation caused by fucoidan.

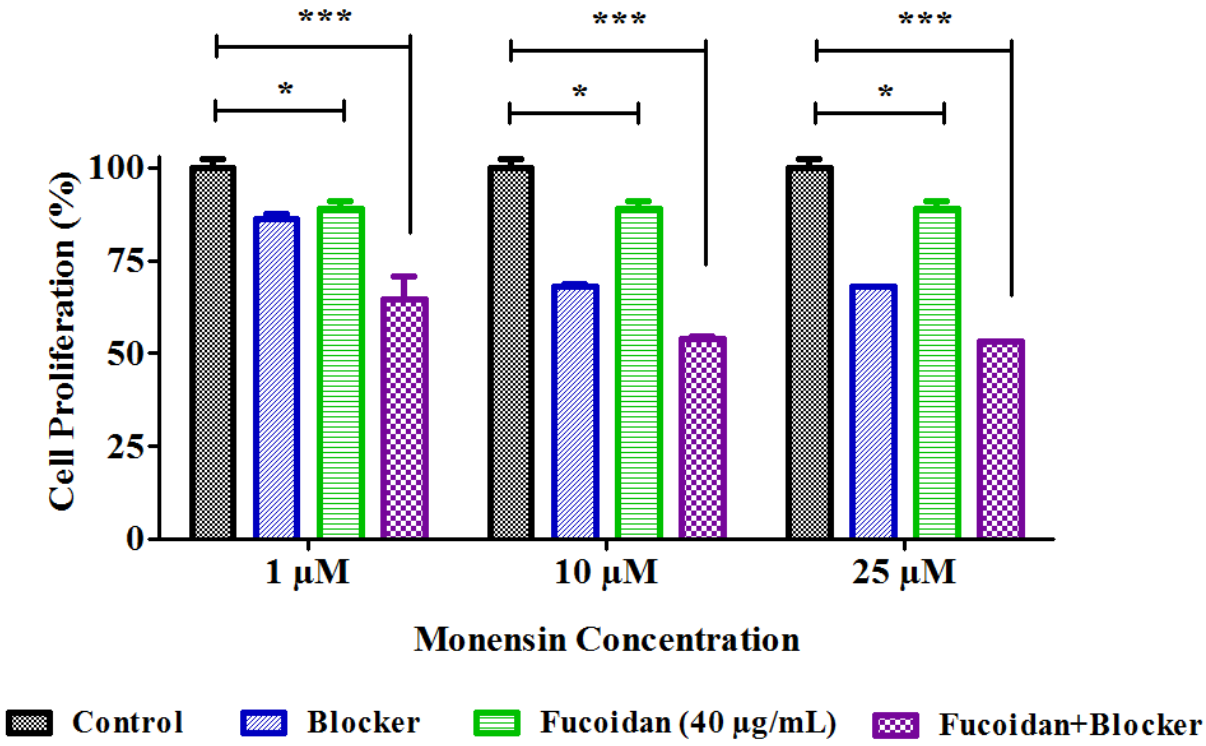


Figure 3.20. WST-8 proliferation assay in NB4 cells pre-treated with endocytosis blocker; monensin. NB4 cells were pre-treated with an endocytosis blocker for 2 hours following treatment with fucoidan for 24 hours. Cell proliferation was assessed using WST-8 proliferation assay. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by two-way ANOVA, followed by Bonferroni post-test.
(***: $p \leq 0.001$, *: $p \leq 0.05$)

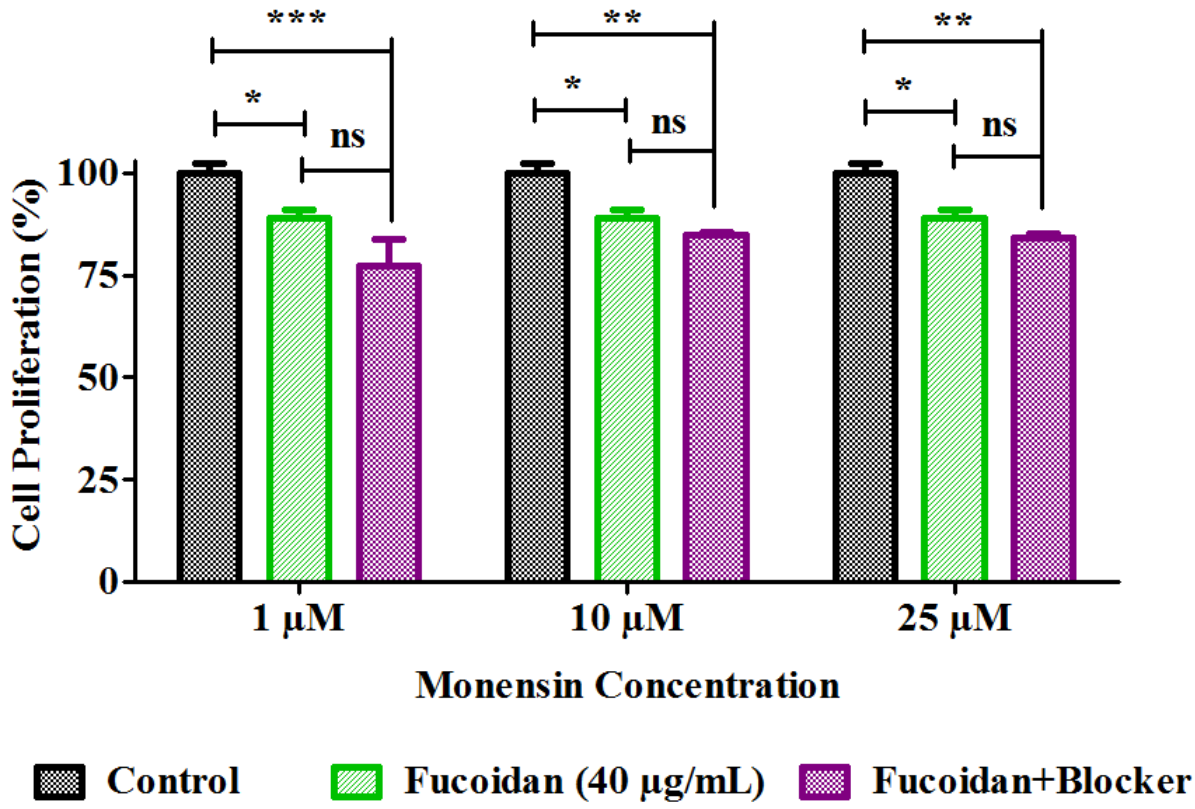


Figure 3.21. WST-8 proliferation assay in cells pre-treated with endocytosis blocker, monensin, after data normalization. NB4 cells were pre-treated with an endocytosis blocker for 2 hours following treatment with fucoidan for 24 hours. Cell proliferation was assessed using WST-8 proliferation assay. The data were normalised according to blocker-only-treated cells. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by two-way ANOVA, followed by Bonferroni post-test.
(***: $p \leq 0.001$, **: $p \leq 0.01$*: $p \leq 0.05$)

3.4 Discussion

Analysis of the tumour inhibitory effect of fucoidan in various types of AML revealed that fucoidan specifically inhibited the growth of the APL cell lines NB4 and HL60. The growth-inhibitory potential of fucoidan in APL cells was mainly due to activation of caspases and inactivation of pro-survival signalling pathways. The data presented here also shows the differing activity of fucoidan from different sources.

3.4.1 Fucoidan Metabolism and Variable Activities of Fucoidan from Different Sources

Despite the promising findings about the anti-cancer effect of fucoidan, there are still challenges impeding utilization of fucoidan in the clinic. The overall review of the literature demonstrates wide variability of the effects of fucoidan in different studies. The differences and the controversies between the results mainly stem from the variable nature of fucoidan (including its structure and molecular weight) depending on its source, amongst other factors.

In this thesis, fucoidan extracts from various sources revealed different activities. The purified fucoidans from two seaweed sources (*U. pinnatifida* and *F. vesiculosus*) supplied by Marinova Pty. Ltd. did not affect cell proliferation and DNA content even at the very high doses of 2 mg/mL. In contrast, the fucoidan from *F. vesiculosus* from Sigma-Aldrich Co. highly inhibited the growth of AML cell lines.

It is well known that the difference in molecular backbone structures of fucoidan extracts can result in differences in their bio-properties. Fucoidan structure differs depends on various factors such as the harvest season, seaweed sources, the parts of the plant, extraction and the purification methods used and the stage of development of the algae. The fucoidan extract from *F. vesiculosus* supplied by Sigma-Aldrich Co. is prepared by dissolving the crude fucoidan in deionised water, acidified to precipitate algenic acid, centrifuged, filtered and lyophilised whereas Marinova Pty. Ltd. supplied fucoidans are extracted using a cold water technique. We believe that differential structural changes observed in the composition analysis of different fucoidans could have been due to different extraction methods.

Sulphate content has the most essential role in bioactivity of fucoidan. The use of de-sulphated and over-sulphated fucoidan with the sulphate content varying from 6% to 41% has shown that

the minimum necessary sulphate content for anti-proliferative activity of fucoidan against CCL39 fibroblast cells is 20% (227). Here, fucoidans from both sources had almost similar amount of near 27% sulphate, as a result sulphate content cannot be the reason for the differential results. The position of sulphate groups in the molecule structure has also a key role in the function of polysaccharides (228), which was not analysed in this study.

Molecular weight is another factor which plays role in fucoidan bioactivity. Here, the MW for purified fucoidan from *U. pinnatifida* from Marinova Pty Ltd. was three times more than that of the fucoidan from *F. vesiculosus* from Sigma-Aldrich Co. (60 kDa vs 20 kDa). Cho and colleagues produced three fucoidan fractions with molecular weights of <5, 5–30 and >30 kDa and compared their anti-cancer activity *in vitro*. Interestingly, the F5-30K revealed the most tumour growth inhibitory effect despites the sulphate amount in F<5K was greater than that of two other fractions (229).

Another significant difference between Sigma-Aldrich Co. and Marinova Pty Ltd. supplied fucoidans was presence of significantly higher amount of uronic acid in the structure of fucoidan from Sigma-Aldrich Co. (more than 4 fold). Uronic acids are acidic carbohydrates, structurally similar to monosaccharaides -except having carboxyl group (COOH) instead of CH_2OH radical in monosaccharaides (230)-, and can be located in the repeating units of polysaccharides (231). Uronic acids are stable structures in the fucoidan molecule and can be prevalently bound to sulphate groups. Different studies have indicated a critical role for uronic acid in the biological activity of polysaccharides (232). In addition, due to the significance of uronic acid in polysaccharide functionality, new studies have attempted to assemble more active molecules by synthesis of uronic acid building blocks (233).

Rosenberg *et al*. (234) compared the structure of highly active and relatively inactive preparations of heparin, a sulphated polysaccharide which has marked structural and functional resemblance to fucoidan (235), and showed that the highly active heparin contained a greater number of uronic acid residues than the relatively inactive substance. In addition, in highly active heparin, a large number of non-sulphated uronic acid residues were located in a restricted region of the molecule or at penultimate positions within the polysaccharide chain, whereas the sulphated uronic acids presented in the main structure. In another study, the anti-tumour activity of 31 different polysaccharides isolated from a brown seaweed against Ehrlich carcinoma was tested in mice. Among those, only L-fucan and fucoidan containing

approximately 10% uronic acid exhibited anti-tumour potential (236). Here, the amount of uronic acid in Marinova Pty. Ltd. supplied purified fucoidan from *U. pinnatifida* was near 1.5% compared to 6% in Sigma-Aldrich Co. supplied fucoidan from *F. vesiculosus*. Although the position of sulphate and uronic acid on fucoidans' structure was not examined in this study, the significantly low amount of uronic acid in purified fucoidan molecule from Marinova Pty. Ltd. might explain its inactivity.

One factor that can make the results challengeable is the presence of 3.9% polyphenol in the fucoidan from Sigma-Aldrich Co. Polyphenol is a phytochemical widely distributed in plants including marine plants such as seaweeds and is reported to have anti-oxidant and cytotoxic effects (237). As purification of the product was not carried out, it is not clear how much the presence of 3.9% polyphenols could have interfered with the cytotoxic effects of the fucoidan.

Another important unanswered question is that whether fucoidan penetrates into the cell and directly performs its activities or remains outside of the cells and exerts its functions through attachment to surface receptors and downstream molecules. To answer to this question, cells were pre-treated with an endocytosis blocker (monensin) to prevent the entrance of fucoidan into the cells. The cell proliferation was then analysed 24 hours after treatment with fucoidan. Upon NB4 cell treatment with monensin (alone or in combination with fucoidan), it was observed that the monensin itself decreased the NB4 cell proliferation. Monensin is a monovalent ion-selective ionophore that prevents the internalization of a variety of ligands. It also acts as a Golgi apparatus plug and interferes with vesicular transport. It is established that monensin decreases cell proliferation and induces cell death through mitochondrial damage and dilatation of the Golgi apparatus in various tumour cells (238). Park *et al.* showed the antiproliferative effects of monensin through cell cycle-related proteins such as p27 in AML cell lines (239). Data obtained in this project is consistent with these findings. Similar to monensin, fucoidan induces apoptosis via different apoptosis and cell cycle related proteins. This may explain the enhanced reduction of cell proliferation in NB4 cells when they were co-treated with both fucoidan and monensin.

The data represented here showed that the endocytosis blocker did not restore the apoptosis mediated by fucoidan. Since the monensin itself had growth inhibitory effect, the combination data were normalised accordingly and yet still endocytosis blockage did not restore the reduced cell proliferation by fucoidan suggesting that fucoidan's action is not associated with its

entrance into the cell. Nevertheless, since hydrophobicity of the molecule plays a crucial role in its transport across the membrane I believe that this result is not strong enough to conclude that fucoidan does not enter the cells and performs its action via surface receptors and downstream molecules. In 2002, Deux *et al*. evaluated the internalisation of LMW fucoidan into rabbit smooth muscle cells (SMCs). Fucoidan was shown to be internalised by endocytosis at 6 h and the number of vesicles containing fucoidan increased in the peri-nuclear region at 24 h. However, they did not observe fucoidan's nuclear internalisation at any time during the study (150). In contrast, Kimura *et al*. investigated the transport of a native fucoidan from *Cladosiphon okamuranus* (MW: 80 kDa) and reported a poor permeation of fucoidan across the human colon adenocarcinoma Caco-2 cell monolayer (151). To investigate this further, it is suggested that the penetration of fucoidan be analysed using high-performance liquid chromatography (HPLC) in whole cell lysate after treatment with fucoidan. This could also be assessed using cell imaging system when an antibody against fucoidan becomes available.

3.4.2 Cytotoxic Effects of Fucoidan on different AML cells

In this chapter, the examination of tumour inhibitory effect of fucoidan in various types of AML showed that fucoidan specifically induced apoptosis in APL cell lines (NB4 and HL60), but not other AML cell lines. HL-60 cells are negative for t(15;17) whereas NB4 cells are positive for t(15;17). The induced cell death was observed in both cell lines indicating that the translocation t(15;17) is not required for fucoidan activity.

Induction of apoptosis is one of the key pathways exploited by anti-cancer agents and is activated through either the extrinsic pathway which is activated by a death receptor located on the cell membrane or the intrinsic pathway which is activated by Bcl-2 family followed by downstream mitochondrial signals. Kim *et al*. and Lee *et al.* reported that fucoidan from a similar source as ours induced tumour cells apoptosis via activation of caspases (167, 240). In contrast, Costa *et al.* demonstrated that the apoptosis mediated by fucoidan (from *Sargassum filipendula*) in Hela cells was caspase-independent (172). Zhang *et al*. however reported that fucoidan from Mozuku increased activation of caspase 9 only and not caspases 7 or 8 in MCF-7 breast cancer cells (160). These results imply that the action of fucoidan is highly dependent on cell type and fucoidan source. In this thesis, fucoidan activated caspase 8 and this was correlated to activation of death receptor DR5 (TNF-related apoptosis-inducing ligand

(TRAIL) receptor II). Caspase 9 was also activated by fucoidan's action and this was correlated with upregulation of the pro-apoptotic molecule Bax. The pan-caspase inhibitor, Z-VAD-fmk, significantly prevented fucoidan-mediated apoptosis, suggesting that caspases are essential in fucoidan-induced apoptosis. Taken together, fucoidan induces apoptosis in APL cells through both intrinsic and extrinsic pathways.

The cyclin dependent kinase inhibitor P21/WAF1/CIP1 is a negative cell-cycle-regulatory protein. Transcriptional activation of P21/WAF1/CIP1, either p53-dependent or independent, inhibits the accumulation of cyclin-CDK complexes and therefore causes G1/S and G2/M arrest. The cell cycle arrest caused by P21/WAF1/CIP1 has also been shown to stimulate the cleavage of caspase 9 resulting in apoptosis within the cell (241). Data presented here demonstrated that fucoidan led to accumulation of P21/WAF1/CIP1 in APLL cells. This is supported by others (163, 166) who have demonstrated the involvement of fucoidan in P21/WAF1/CIP1 upregulation in prostate and bladder cancer cell lines, respectively.

MAPK and PI3/AKT are key components of the signalling pathways involved in cell proliferation and transmit signals through enzymes such as Raf, MEK, ERK and AKT (155). Since fucoidan has been shown to target cell survival and apoptosis processes, it is hypothesised that the mechanism of action is inducing changes in the activation of signalling pathway molecules. Here, expression and activation of two kinases involved in these key signalling molecules were evaluated; ERK and AKT in the MAPK and P13K pathways respectively. Fucoidan reduced activation of both enzymes through hypo-phosphorylation, but it did not alter their total expression levels.

PI3K/AKT is a signal transduction pathway whose activation mediates multiple biological processes such as cell survival, growth and proliferation and inhibits apoptosis. AKT is one of the key enzymes in this pathway and, as mentioned, fucoidan suppressed AKT activation, leading to cell death. Our findings regarding the observed inactivation of AKT are similar to those of others (171, 242). It has also been reported that AKT over-activation is associated with drug resistance, tumour cell survival and migration (243). AKT activation occurs following phosphorylation of two different residues: Thr308 (in the activation loop) and Ser473 (in the C-terminal hydrophobic motif). Activation of Thr308 and not Ser473 correlates with poor prognosis in AML (244). The analysis of the phosphorylation of AKT in both Thr308 and Ser473 residues indicated that fucoidan did not alter the phosphorylation of AKT on the Ser473

site in APL cell lines, but it down-regulated the phosphorylation of AKT on the Thr308 residue. This is the first study that has reported a selective effect of fucoidan on AKT phosphorylation sites.

In this study, fucoidan markedly inactivated ERK. Studies by Patel *et al*. and Aisa *et al*. also showed that the anti-mitogenic property of fucoidan is mediated by hypo-phosphorylation and deactivation of ERK1/ERK2 in vascular smooth muscle cells and in human lymphoma cells, respectively (155, 182). In contrast, Hsu and colleagues proposed that fucoidan causes ERK activation rather than inactivation (190). It is known that the ERK signalling pathway is complex and induces a range of different responses including cell proliferation, differentiation, migration and even apoptosis depending on cell type, the type of stimulus and duration of activation. For instance, it is generally believed that the ERKs progress cell proliferation in most types of cells, while in some studies, the ERKs have shown pro-apoptotic (and not mitogenic) influences on renal cell lines (245). In contrast to this thesis, Jin and colleagues reported activation of ERK in NB4 and HL60 cells (170). It should be highlighted that duration of activation and timing are the main variables in signal transduction pathways studies. ERK activation was measured after 24 hours but in the study by Jin *et al*., the ERK activation was measured after only 15 minutes.

In contrast to APL cell lines, fucoidan did not induce apoptosis in the AML cell lines KG-1a and K562. This cell-type specific growth inhibitory activity of fucoidan has been previously reported. Fukahori *et al*. examined 6 different hepatocarcinoma cell lines revealing selective and differing inhibitory effects of fucoidan (169). Cell lineage and maturation stage of haematopoietic cells are governed by altered regulation and differential expression of sets of genes in different pathways and these differences are likely to affect the cell response to drugs. The $CD34^{+}/CD38^{-}$ cells isolated from both K562 and KG-1a cell lines are reported to have less phosphorylated p38 MAPK than those isolated from human cord blood (246). It has been suggested that this reduced phosphorylation of p38 may reduce the sensitivity of these cell types to activation of apoptosis. Xiao *et al*. showed that modification of p38 through over-phosphorylation induced growth arrest and apoptosis in both K562 and KG-1a cell lines (246). Previously published studies have also reported that fucoidan induces apoptosis in monocytic U937 cells through p38 activation (247). Here, it is hypothesised that lower phosphorylation

of p38 in K562 and KG-1a cell lines could possibly protect them against the anti-tumour activity of fucoidan.

In agreement with data obtained here, Jin *et al.* also reported that fucoidan does not induce apoptosis in K562 cells (170). However, the cell proliferation study in this project revealed that fucoidan slightly decreased proliferation of K562 cells after 48 hours. To investigate this further, the DNA content was analysed and a marked increase in G0/G1 cell population was found in treated K562 cells. This indicates that fucoidan induces cell cycle arrest without switching on the apoptosis pathway in this cell type. Unlike the NB4, HL60 and K562 cell lines, the undifferentiated AML cell line KG-1a was resistant to the growth inhibitory function of fucoidan. In DNA content analysis, fucoidan induced neither cell cycle arrest nor sub G0/G1 dead cell population.

In summary, it is concluded that fucoidan's action against tumour cells is cell-type specific and further that APL cells showed higher response to fucoidan. Fucoidan was found to reduce the activation of ERK1/2 and AKT in APL cells and induce the activation of caspases 8, 9 and 3 where fucoidan's action was attenuated using a pan-caspase inhibitor.

CHAPTER FOUR

Prophylactic Anti-Tumour Activity of Fucoidan in AML

4.1 Introduction

Seaweed, a common food source in Asian countries, is rich in bioactive metabolites such as poly-phenols, sulphated polysaccharides (fucoidan and laminarin) and primary carotenoid (fucoxanthin). Epidemiological and experimental evidence supporting the protective effect of these active ingredients against cancer development have been growing in the last three decades (248-251). Epidemiological studies have supported this with reports of low risk and mortality from certain types of cancers in populations with daily consumption of seaweeds.

Japanese women with regular seaweed consumption are reported to have 1/3 the rate of premenopausal and 1/9 the rate of postmenopausal breast cancers (252). The risk of cancer between seaweed- to non-seaweed-consuming American postmenopausal women has been also evaluated in a clinical trial (253). In this study, consumption of 5 g / day brown seaweeds for duration of one month resulted in 50% reduction in systematic concentration of the Uri-kinase receptor (uPAR) whose overexpression is associated with higher risk of cancer and tumour cell invasion and metastasis (254).

The anti-cancer activities of the dietry seaweed have been further explored. Teas *et al*. reported a significant delay in adenocarcinoma development in female rats fed with dietary Seaweed (*Laminaria*) (188). In 1991, Furusawa *et al.* investigated the prophylactic activity of a dietary seaweed *Undaria pinnatifida* extract, referred as Viva-Natural, against Rauscher murine retrovirus-induced erythroleukaemia (255) which is characterised by development of splenomegaly within 2-3 weeks after virus inoculation. They pre-treated mice with IP administration of seaweed extract on day 3 or day 1 or 1 hour prior to virus inoculation and analysed the leukaemia progression on day 21. Pre-treatment on 3 days prior to inoculation significantly inhibited the growth of leukaemic cells. Pre-treatment with the extract was not effective on tumour growth when administrated on day -1 or 1 h before virus inoculation (256).

It has been subsequently found that fucoidan is the main brown seaweed ingredient which possessed tumour inhibitory effects. Despite the reported anti-tumour activity of fucoidan in different types of cancers, very little *in vivo* work has focused on protective activity of fucoidan against cancer development; but rather has focused on its therapeutic effects after tumours have been established in animals.

Several theories have been proposed about prophylactic activity of fucoidan. One hypothesis is its anti-oxidant activity. The association between oxidative stress involving active states of oxygen and tumourigenesis process is well established (257). Oxidative stress agents, or free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (e.g. hydrogen peroxide and nitric oxide) are highly reactive chemicals that harm cells through damaging lipids, proteins and DNA (258). Antioxidants function as free radical scavengers and protect cells against oxidative damages. Rocha de Souza *et al*. study has shown that fucoidan exhibits the highest antioxidant and superoxide radical scavenging activity amongst different polysaccharides derived from brown seaweeds (259). *In vitro* and *in vivo* evidences have also indicated that fucoidan prevented the free radical synthesis, reversed the oxidative damages and restored the increases in free radicals in the spleen tissue caused by oxidative agents (260).

The second hypothesis arises from the demonstrated immunomodulatory activity of fucoidan (261). Fucoidan enhanced the anti-tumour immune response through various pathways including increased phagocyte activity of macrophages (262), enhanced functional maturation of dendritic cells (263), and increased number of NK and cytotoxic T-cells (185). It also modulated the production of cytokines by immune cells. Interferon-γ (IFN-γ) is a cytokine that promotes the innate and adaptive immune responses and has a critical protective role against tumour development. Fucoidan markedly increased the intracellular level of IFN-γ in NK cells *in vitro* (264), and elevated secretion of IFN- γ in serum *in vivo* (187).

The aim of this chapter was to investigate the prophylactic activity of fucoidan against human acute myeloid leukaemia development in mice. To determine the immunomodulatory activity of fucoidan was the second aim of this chapter. To address this, the splenic natural killer cell cytotoxicity activity was examined in mice that were orally pre-treated with fucoidan.

4.2 Experimental Design

4.2.1 Analysis of Prophylactic Activity of Fucoidan *in Vivo*

The acute promyelocytic leukaemia NB4 cells were selected as they were sensitive to fucoidan. NB4 cells provide a suitable model for xenograft human APL. Five to seven week old BALB/c athymic nu/nu mice were randomly divided into two groups; the control group (n=8) and the fucoidan group (n=9) (Table 4.1). The animals in the fucoidan group were daily fed with 150 µg/g b.w. fucoidan from *F. vesiculosus* (Sigma-Aldrich Co.) mixed with peanut butter for two weeks (see Section 2.9.4.1). The control group was fed with the same amount of peanut butter mixed with vehicle (distilled water). The animal's body weight was measured daily in both groups until the end of the study and doses were calculated daily according to body weight. After 14 days, NB4 cells were injected subcutaneously in the right flank of each mouse and the xenograft tumour appearance and growth were monitored daily. When the transplanted tumour volume reached 1000 mm^3, the mice were humanely euthanised (see Section 2.9.7). Tumour volume doubling time and tumour growth rate were calculated for each mouse.

Table 4.1. The Number and Gender of Mice Used for Prophylactic Study

	Control	**Fucoidan**
Female	4	4
Male	4	5
Total	**8**	**9**

4.2.1 Analysis of Immunomodulatory Activity of Fucoidan *in Vivo*

To investigate the underpinning mechanism of the prophylactic activity of fucoidan on tumour growth, the immunomodulatory effect of fucoidan and its role in mice's NK cell function was investigated. To do this, twelve 5-7 week old male BALB/c athymic nu/nu mice were randomly divided into two groups; the control group (n=6) and the fucoidan group (n=6). Animals were fed with 150 µg/g b.w. fucoidan (Sigma Co.) or distilled water in peanut butter (as described in Chapter 2). After 14 days, the mice were humanely euthanised with inhalation of CO_2 and the cytotoxic activity of spleen NK cells was investigated using flow cytometry (see Section 2.9.10).

4.3 Results

(This section has been substantially published in Atashrazm et al. 2015 (221))

4.3.1 Oral Fucoidan Delayed the AML Appearance and Growth in Mice

To analyse the protective activity of fucoidan on leukaemia development, 17 mice were pre-fed with fucoidan followed by tumour cell injection and the effect of orally administered fucoidan on subcutaneous xenograft tumour development was examined. Three out of eight female mice including 1 in control group and 2 in fucoidan group did not develop tumour for up to 50 days. As a result the experimental animals were limited into 14 mice: 7 in each control and fucoidan group. No signs of toxicity were observed in mice fed with fucoidan.

No significant difference was observed in body weight in animals and it slightly increased in both control mice and fucoidan mice; mean ± SD of body weight gain was 3.25±1.65 g and 3.85±1.95 g in control and fucoidan groups, respectively (see Appendix D).

Significant anti-tumour activity was observed in mice fed with fucoidan compared to the control ($p \leq 0.001$) (Figure 4.1). Following inoculation with NB4 cells the subcutaneous tumour mass appeared in less than 10 days in 5 out of 7 mice in the control group and 2 out of 7 mice in the pre-treated fucoidan group. The mean of doubling time for the tumour mass was 1.8 days in the control group compared to 4.64 days in the fucoidan group. The growth rate which is the number of volume doublings that occurred per day was also calculated. The mean of tumour growth rate was 0.37 in the control group and 0.23 in the fucoidan groups.

The tumour size reached the end point in less than 12 days after tumour appearance in all mice in the control group compared to 1 mouse in the fucoidan group. Figure 4.2 shows the tumour mass at day 19 after tumour cell injection in two representative mice from fucoidan and control groups. Figure 4.3 compares the tumour volume in both groups at four-day intervals for up to 12 days when all mice in control group were sacrificed.

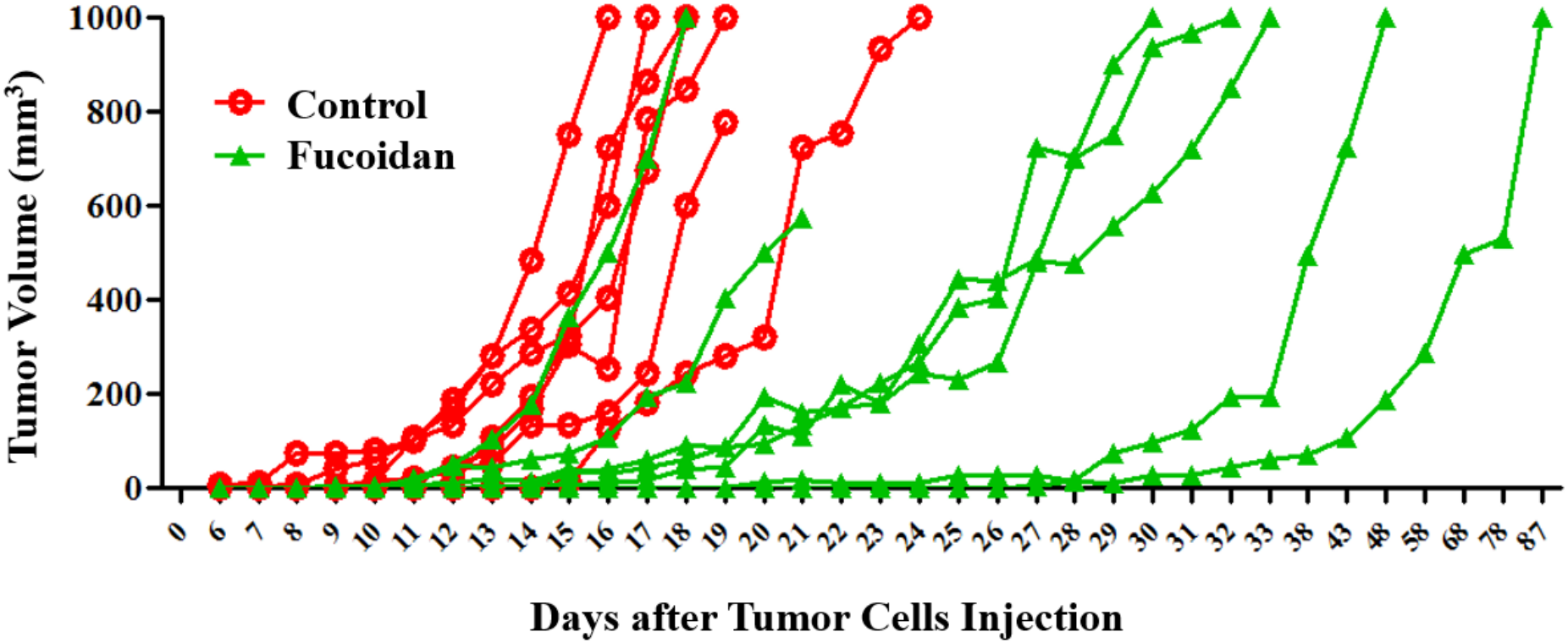


Figure 4.1. Tumour appearance and growth in mice fed with vehicle or fucoidan. Mice were fed with fucoidan or vehicle for 14 days (n=7/group). NB4 cells were injected into mice and tumour volume was measured daily. Each line represents the daily tumour volume in one mouse in fucoidan group (green) and control group (red).

* Due to tumour rupture, two mice (1/group) were sacrificed before the tumour size reached the end point.

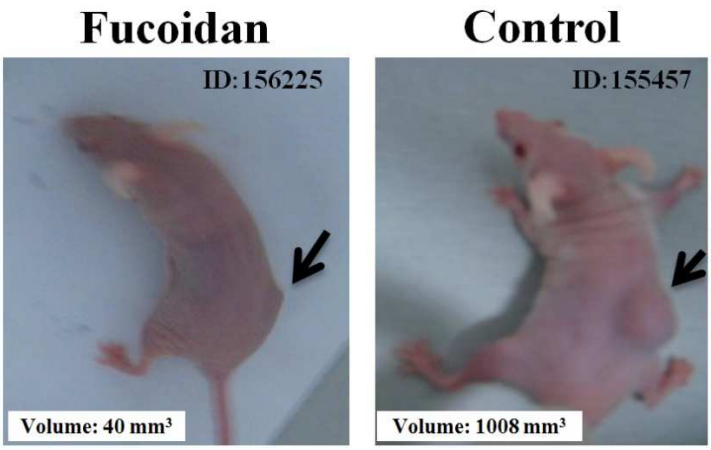


Figure 4.2. Representative tumour mass at day 19 after NB4 cells injection. Mice were either fed with fucoidan or vehicle for 14 days. NB4 cells were then injected SC in the mice's right flank. The images demonstrate the tumour mass in one mouse in each group at day 12 after injection.

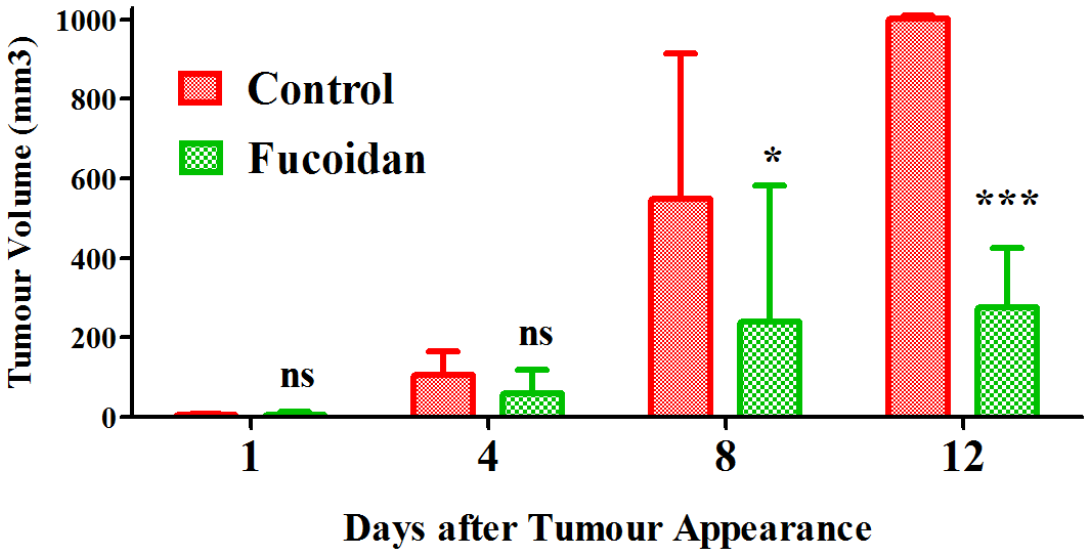


Figure 4.3. tumour Volume in fucoidan and control groups at 4-day intervals. Mice were fed with fucoidan or vehicle for 14 days (n=7/each group). NB4 cells were injected into mice and tumour volume was measured daily. The tumour mass in all control mice reached the end point within 12 days after the tumour appearance. The columns and bars represent mean ± SD (***: $p \leq 0.001$, *: $p \leq 0.05$).

4.3.2 Fucoidan Increased Splenic NK-cell Activity *in Vivo*

Given our observation and the known immunomodulatory activity of fucoidan, it was hypothesised that fucoidan increases the cytotoxic activity of splenic NK cells when it is orally administrated. Twelve additional mice were fed with either fucoidan or distilled water (6 in each group) for 14 days and the mice were humanely euthanised and their spleen was removed. The viability of isolated splenic mononuclear cells (MNCs) was assessed using trypan blue exclusion and was more than 90% for all animals.

To measure the ability of MNCs to lyse the NK cell-sensitive YAC-1 cells, the target YAC-1 cells were first labelled with the fluorescent dye CellTrace™ Violet (CTV). As shown in Figure 4.4A, YAC-1 cells 100% labelled with CTV was established before being used for the experiment. The CTV^+ YAC-1 cells were then combined with effector MNCs at effector cells to target cells ratios of 3:1, 6:1, 12:1, 25:1, 50:1 and 100:1. Each sample was plated in triplicate and a control YAC-1 cell sample was included. After 4 hours incubation, the dead YAC-1 cells were measured using PI staining with flow cytometer. The amount of spontaneous dead YAC-1 cells was measured in YAC-1 control wells only (Figure 4.4B). To calculate the specific YAC-1 cell lysis by NK cells, the amount of spontaneous dead YAC-1 cells in control wells was subtracted from the results of the experiments (Figure 4.4C). The results for one representative mouse in each group are shown in Figures 4.5 and 4.6.

As shown in Figure 4.7, the cytotoxic activity of NK cells from the fucoidan group was higher than that of the control group in all six effector to target cells ratios ($p=0.3$). The difference was statistically significant at 50:1 and 100:1 effector:target cells ratio ($p\leq0.05$, $p\leq0.001$ respectively). This result suggests that consumption of fucoidan can modulate the innate immune system by increasing the cytolytic activity of NK cells.

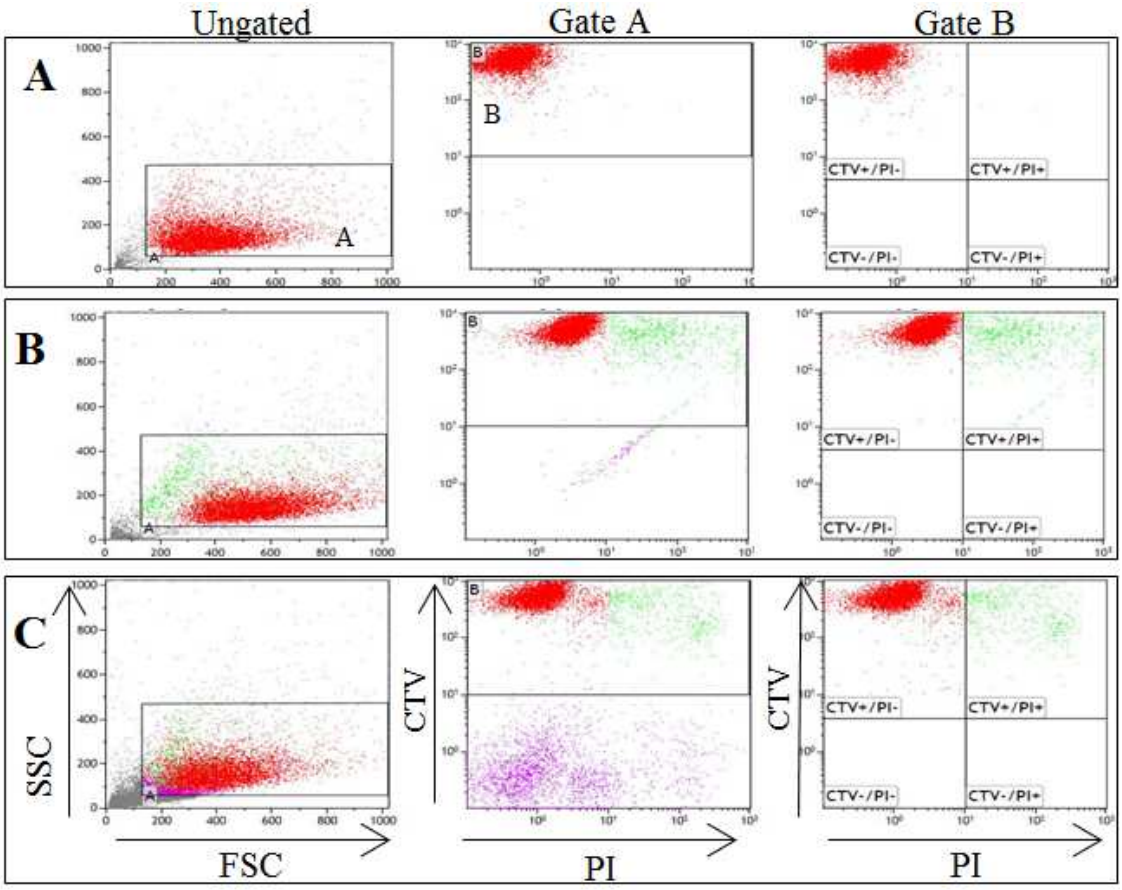


Figure 4.4. Flow cytomety histograms for NK cell cytotoxicity assay in one representative experiment. The YAC-1 cells were labelled with CTV and used as targets for splenic NK cells as effectors. Left histograms show cell population based on size (FSC) and granularity (SSC). Debris was excluded and the remaining cells were gated as "Gate A". Middle histograms show PI versus CTV in "Gate A" population. The CTV^+ cells were gated as "Gate B". Right histograms show PI versus CTV in "Gate B" population. Results from this histogram were used for calculation.

A) "CTV-labelled target cells only" without PI. This control was used to ensure all target cells were labelled with CTV. As shown in the middle graph, all YAC-1 cells were 100% labelled with CTV. The CTV^+ cells were gated and used for NK cell-mediated cytotoxicity analysis. **B) "CTV-labelled target cells only" with PI.** The 0:1 effector to target cells were used as the minimum control cells. The amount of PI^+ dead YAC-1 cells (green population) was calculated as spontaneous cell death. **C) CTV-labelled target cells plus effector cells.** Splenic mononuclear cells obtained from each mouse were added to the target cells. The amount of PI^+ dead YAC-1 cells (green population) was calculated. The percentage of spontaneous dead cells was subtracted from these results.

Red population: live target cells (CTV^+/PI^-), **Green population**: dead target cells (CTV^+/PI^+), **Purple population**: effector cells (CTV^-)

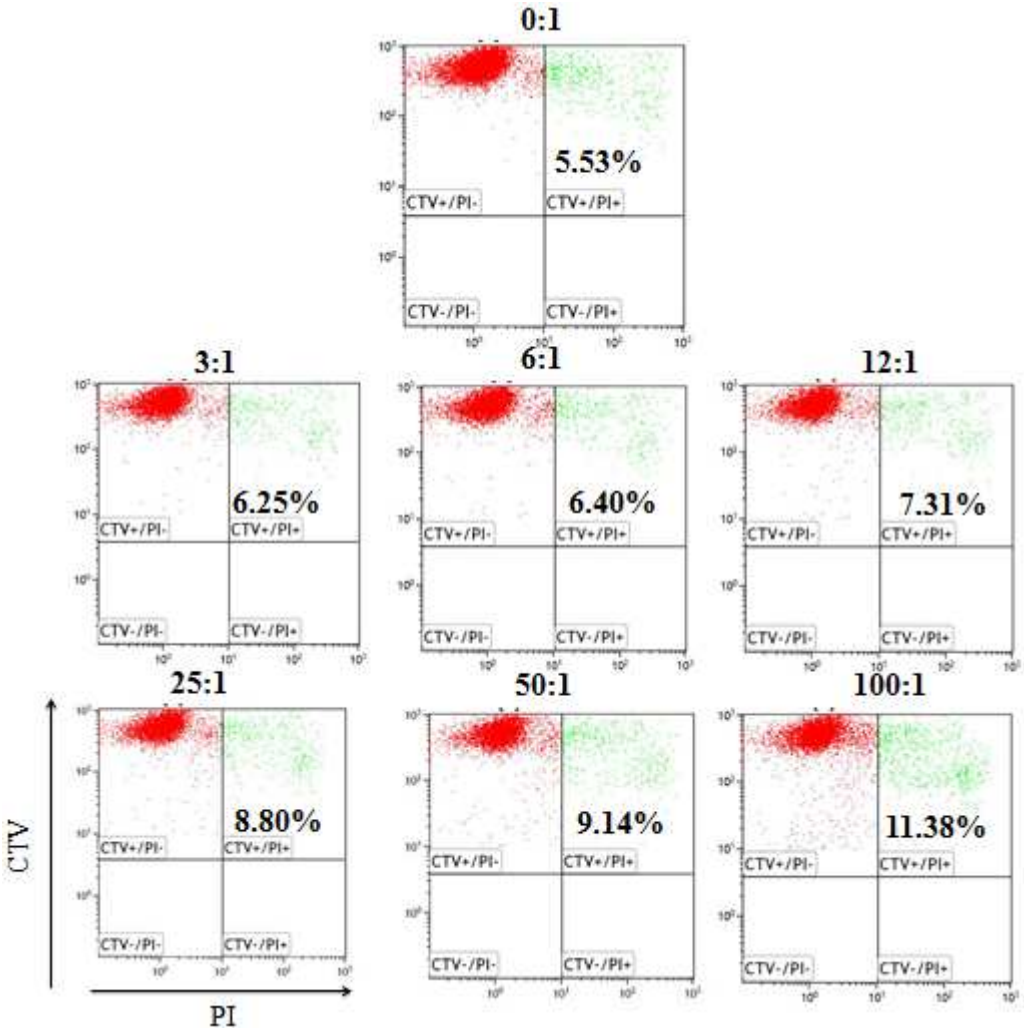


Figure 4.5. NK cell cytotoxicty assay in one representative mouse in control group. Mice were fed with vehicle for 14 days (n=6). Splenic mononuclear cells were isolated and NK cell cytotoxicity was analysed using flow cytometry. The CTV^+ YAC-1 cells were used as targets for splenic NK cells (effectors). The dead YAC-1 cells were analysed with PI staining. Each histogram demonstrates the live (red) and dead (green) YAC-1 cells in various dilutions of effector:target. To measure the spontaneous cell death, minimum control cells (0:1) containing target cells only were prepared. Results represent one of three replicates in 1 out of 6 mice.

Red population: CTV^+/PI^- (live target cells), **Green population**: CTV^+/PI^+ (dead target cells)

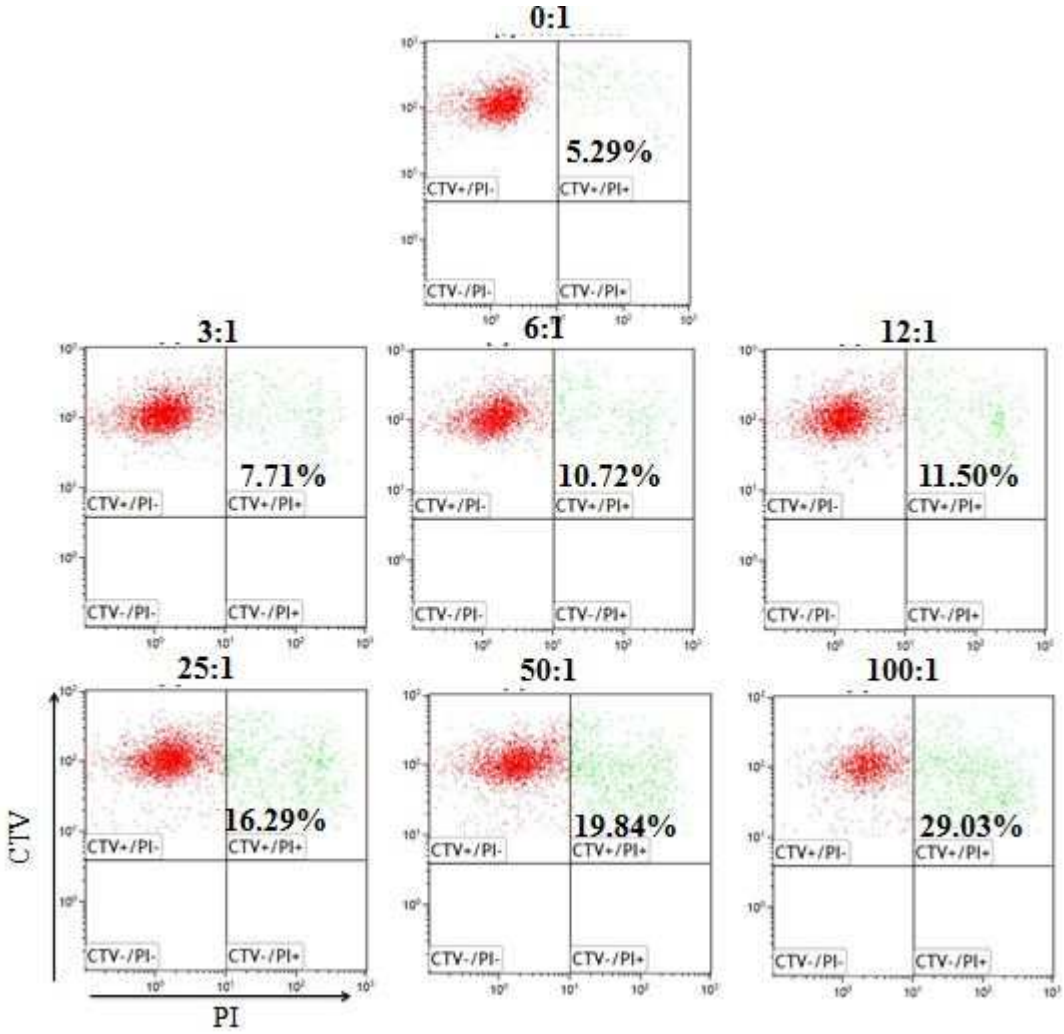


Figure 4.6. NK cell cytotoxicty assay in one representative mouse in fucodian group. Mice were fed with fucoidan for 14 days (n=6). Splenic mononuclear cells were isolated and NK cell cytotoxicity was analysed using flow cytometry. The CTV^+ YAC-1 cells were used as targets for splenic NK cells (effectors). The dead YAC-1 cells were analysed with PI staining. Each histogram demonstrates the live (red) and dead (green) YAC-1 cells in various dilutions of effector:target. To measure the spontaneous cell death, minimum control cells (0:1) containing target cells only were prepared. Results represent one of three replicates in 1 out of 6 mice.

Red population: CTV^+/PI^- (live target cells), **Green population**: CTV^+/PI^+ (dead target cells)

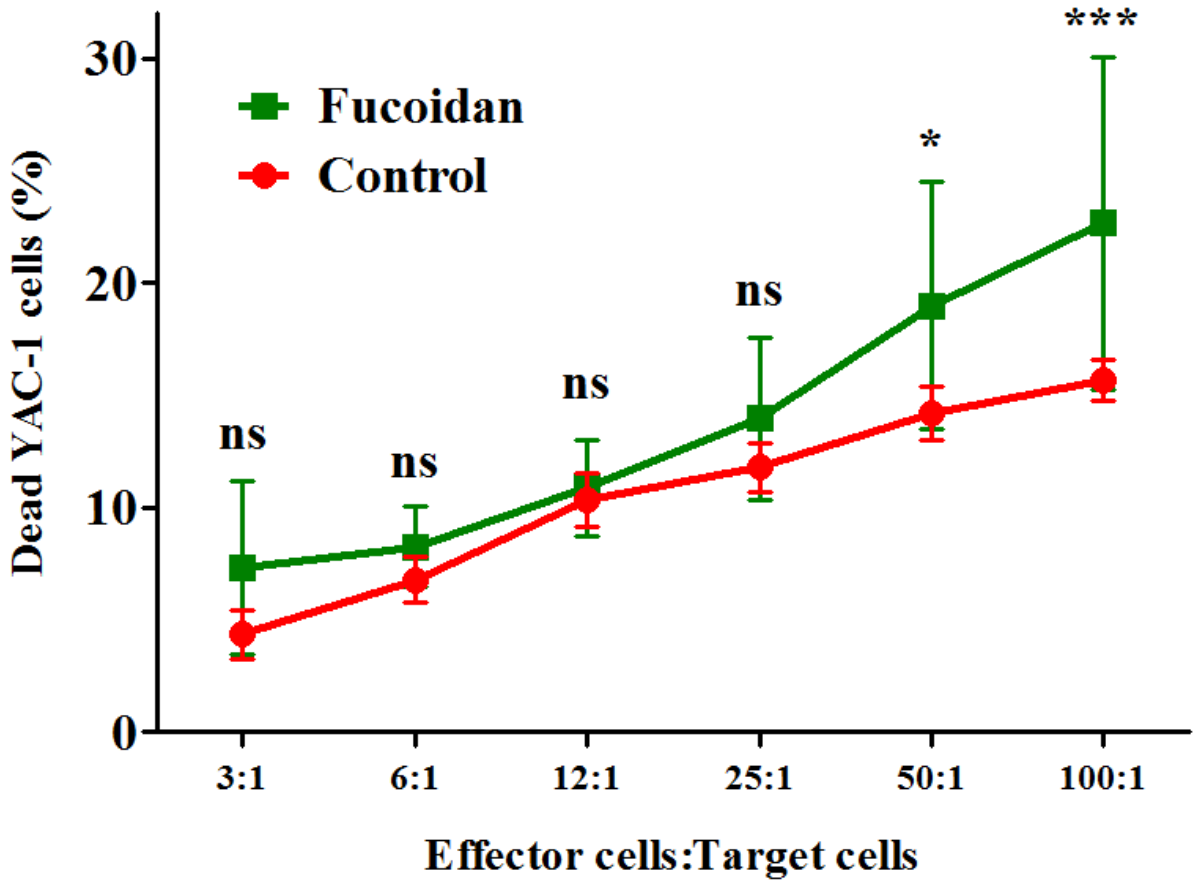


Figure 4.7. Splenic NK cell cytotoxicity. Percentage of dead targets cells (YAC-1 cells) in mice fed with fucoidan (green) and control mice (red) were calculated (n=6/group). Data represent mean ± SD. Statistical significance was determined by two-way ANOVA, followed by Bonferroni post-test (***: $p \leq 0.001$, *: $p \leq 0.05$).

4.4 Discussion

Data in this chapter presents evidence that fucoidan has protective effects against development of acute promyelocytic leukaemia in a xenograft mouse model. APL appearance and growth were significantly delayed in mice that were pre-fed with fucoidan. This was possibly through modulation of immune system as fucoidan enhanced cytolytic activity of NK cells in mice.

4.4.1 Protective Effect of Fucoidan against AML development in Mice

A large number of epidemiologic, animal and laboratory studies indicate that dietary factors play a key role in cancer prevention and progression. Several studies have associated consumption of seaweeds containing fucoidan with low risk of cancers (265). The preventive effect is highly related to the anti-inflammatory and immunomodulatory activities of this component. Herein, the prophylactic effects of fucoidan on an acute myeloid leukaemia mouse model were examined. Development of APL following NB4 cell inoculation was investigated in mice that were pre-fed with fucoidan and it was observed that fucoidan significantly protected mice against APL development.

Few studies have shown that fucoidan inhibits the neoplastic transformation. In a study by Teas *et al*., a group of female Sprague-Dawley rats were fed with dietary seaweed (laminira) for 34 days and then were given carcinogen 7,12-dimethylbenz(a) anthracene. Feeding was continued per day for the entire period of the study. The animals were monitored for 26 weeks and the rate of tumour development was examined. Experimental rats showed a significant delay in tumour appearance; the median time from carcinogen administration until first tumour appearance was 19 weeks in the seaweed-fed rats and 11 weeks in the control rats (253). The delayed tumour appearance observed in Teas *et al*. study is similar to results in this Chapter. Although mice were treated for a shorter period of time than their study and feeding was not continued after tumour inoculation, fucoidan still could protect the animals against tumour development.

In a recent study by Huang *et al*, oral administration of fucoidan prophylactically alleviated cachectic symptom of lung carcinoma (266). In their study inoculation of Lewis lung carcinoma cells in mice resulted in body weight loss in tumour-bearing animals compared to non-bearing-tumour control mice. But mice orally fed with fucoidan (1 or 3 mg /mouse) had significant increase in the body weight compared to tumour-bearing mice receiving water. In

this thesis, there was no significant difference in body weight, and animals in both control and fucoidan groups slightly gained similar amount of weight during the study. Difference between these results and those of Huang *et al*. study may be due to various tumour model used as they established systematic tumour through IV injection of tumour cells while the model developed here was a subcutaneous transplanted tumour.

Data presented in this chapter of thesis showed that the utilised animal model had some limitations as 37.5% of female mice (3 out of 8) did not develop tumours. Prior to commencing the main experiments and to investigate whether xenograft transplantation with human NB4 cells would be feasible in nude mice, 5 female mice were obtained and injected subcutaneously with NB4 cells (Data not shown). It was demonstrated that 40% of mice (2 out of 5) from that batch also did not develop tumour for up to 3 months. In contrast, the xenograft transplantation in male mice was 100% successful and all mice grew tumour within 7-14 days. Due to these observations, the experiments evaluating the immunomodulatory activity of fucoidan were conducted on male mice only. The reason behind the tumour establishment failure in female mice is not clear to us. Several studies have used female nude mice for establishment of different types of cancers including NB4 cells (267, 268). In a report by Zhang *et al*., NB4 cell transplantation into nude mice was unsuccessful in their unpublished data (269). However, they did not report whether or not the unsuccessful tumour growth was sex dependent. The hormone-rich environment presenting in females might be one of the factors that affected the NB4 cell growth. Moreover, some nude mice have been shown to have extra-thymic T-cells, which can affect the tumour development. Hence optimization assays are suggested for cancer research using xenograft transplantation in nude mice. Moreover, it is suggested that using NOD-SCID mice (non-obese diabetic-severe combined immunodeficiency) might be a better choice for xenograft models as they lack T-cells, B-cells and NK cells.

4.4.2 Immunomodulatory Activity of Fucoidan *in Vivo*

To evaluate the mechanisms underpinning the delayed tumour growth in mice pre-fed with fucoidan, the immune system was examined in mice as it is pivotal for cancer development.

In a recent study, Zuo and colleagues examined immune system in mice orally fed with fucoidan (270). They examined the intestinal tissue samples from mice being fed with fucoidan and reported that dietary fucoidan markedly increased the mRNA level of IL-6 and IL-10. In

addition, a significant increase in IgA expression was observed in intestinal samples, which probably occurred upon upregulation of the aforementioned cytokines.

Recently, Zhang *et al.* showed that IP injection of 50 µg/g b.w. fucoidan significantly increased the percentage and cell number of NK cells in C57BL/6 mice (264). Here, we also investigated the effect of fucoidan on NK cells which are the key component of body's innate immune system. Although, the focus of this thesis was on the effects of fucoidan on NK cell function.

Although athymic nude mice lack T-cells, they have functional NK cells. NK cells are a subset of lymphocytes, characterised by their ability to kill tumour cells spontaneously. Data presented showed that the cytolytic activity of NK cells in mice that were fed with fucoidan for two weeks significantly increased compared to the control group. This was shown by the amount of dead target cells in fucoidan group which was higher than that of the control group in all six effector:target cells ratios with a maximum of 1.6 fold at 100:1 ratio. Maruyama *et al*. have similarly reported higher NK cell activity in fucoidan-treated mice. Although, they report 2.3 fold higher than the control group which could be as a result of the different route of administration as they injected 50 µg/g b.w. of fucoidan intraperitoneally (271).

NK cells mediate their anti-cancer function through cytokine production and also by regulating cytokine production of other immune cells. IFNγ is a major anti-cancer cytokine produced by immune cells such as T-cells and NK cells, and itself modulates NK cell function. A clinical trial has shown that the amount of IFNγ producing immune cells increased to 114% in individuals given a fucoidan mix orally for 30 days. In a recent *in vitro* study, fucoidan treatment activated NK cells and stimulated the production of IFN-γ by them (264). These data support the involvement of activated NK cells –and potentially through increased IFNγ- in delaying the tumour growth in the *in vivo* experiments in this thesis.

In conclusion, it was found that consumption of fucoidan even over a very short time period could protect the animals against APL development and growth and suggested that fucoidan intake has a prominent effect to enhance the immune cell activity and to eliminate the tumour cell development.

CHAPTER FIVE

Investigation of Possible Synergistic Effects of Fucoidan and ATO in APL Treatment *in Vitro* and *in Vivo*

5.1 Introduction

Although therapeutic strategies using ATRA plus anthracycline-based chemotherapy increases the cure rate in majority of new cases of APL, potential short-term and long-term side effects are reported (272). Hyper-leucocytosis and differentiation syndrome (DS) are some short-term sequelae (273), and extra-medullary diseases at the recurrence (274), secondary cancers such as myelodysplastic syndrome (275), and delayed cardiomyopathy (276) are examples of the long term side effects.

Arsenic trioxide (ATO) is the most active single agent in APL treatment. Single administration of ATO induces complete remission in approximately 85-90% of the patients (277, 278). Currently, ATO is administrated for refractory or relapsed APL, or older patients who are unable to tolerate anthracycline-based therapies (38). However, clinical trials are under way to evaluate if revised strategies incorporating ATO in the treatment of newly diagnosed cases of APL could possibly prevent the above mentioned complications caused by ATRA+chemotherapies.

ATO acts through multiple mechanisms. It induces apoptosis, inhibits proliferation and angiogenesis and stimulates cell differentiation (279). Interestingly, ATO has a dose-dependent dual effect on abnormal promyelocytes; at high concentrations of 0.5-2 µM, ATO induces apoptosis in malignant promyelocytes, while at low doses of 0.1-0.5 µM, it induces partial differentiation (32). Apoptosis is mediated by down regulation of the anti-apoptotic molecule Bcl-2 expression both at mRNA and protein level, disruption of mitochondrial transmembrane potentials ($\Delta\Psi m$), induction of accumulation of ROS and modulation and degradation of the PML-RARα hybrid protein (280).

Administration of ATO has been linked with several mild to moderate haematologic and non-haematologic side effects. ATO is reported to induce leucocytosis in about 50% of the cases and also mild to severe hepatic toxicities in a large number of the patients (281, 282). Similar to ATRA, its usage can cause development of the potentially fatal complication, DS, characterised by fever, acute respiratory distress, acute renal failure and potentially death (273). In spite of its high efficacy in induction of complete remission in a majority of the patients (283), the 2-year disease free survival remains quite low (42%) in refractory patients (282).

Therefore, it is of interest to develop complementary treatment strategies which increase the efficacy of current treatment while minimizing the toxicities.

This dissertation has outlined the potential advantages of natural products, and the growing interest in their anti-cancer activities. Due to their multi-functional properties, natural products are also being tested as adjuvants for use in synergy with chemotherapeutic agents.

In recent years, there is an emerging body of work investigating the combined effects of fucoidan with standard anti-tumour agents. For example, fucoidan from *Saccharina cichorioides* was reported to synergise with the anti-tumour activity of low dose resveratrol on the invasive and highly motile HCT 116 colon cancer cell line (195). In a xenograft transplantation study, the effect of fucoidan alone or in combination with cyclophosphamide was examined on tumour growth. Nine days after the injection of Lewis lung carcinoma cells into C57BL/6 mice, fucoidan from *Fucus evanescens* was administered to animals alone or combined with cyclophosphamide. The fucoidan group showed marked anti-tumour (33% tumour growth inhibition) and anti-metastatic (29% reduction of the number of metastases) activities. Although fucoidan did not exhibit a synergistic effect with cyclophosphamide on primary tumour growth, but significantly decreased the lung cancer cell metastasis (284).

In Chapters 3 and 4 of this thesis, fucoidan was shown to possess antiproliferative activities in human APL cell lines both *in vitro* and *in vivo*. It is postulated that the induction of apoptosis by ATO is via mitochondrial dysfunction and further cleavage of PARP1 which is a crucial enzyme involved in DNA repair (285). In Chapter 3, fucoidan was shown to induce apoptosis through a caspase-dependent mechanism and further inactivation of PARP-1 in the acute promyelocytic leukaemia NB4 and HL60 cell lines. Both fucoidan and ATO cleave PARP-1 but through two different pathways, therefore it was hypothesised that the combination of these two agents could synergistically enhance apoptosis in APL cells and that fucoidan may potentiate the anti-tumour activity of the APL standard treatment ATO.

5.2 Experimental Design

5.2.1 The synergistic Effect of fucoidan with ATO in APL Cell Apoptosis *in Vitro*

The chromosomal t(15;17) translocation positive human APL cell line NB4 was treated with low concentration of fucoidan from *F. vesiculosus* (10 and 20 µg/mL) alone, ATO alone (0.25, 0.5, 1 µM) or combination (see Section 2.2). After 48 hours, cell proliferation and apoptosis were analysed using WST-8 proliferation, Annexin V/PI, DNA fragmentation and cell cycle assays (see Sections 2.3 to 2.6).

The non-APL Kasumi-1 AML cell line was used as control and similarly treated as described above for up to 96 hours. Cell proliferation was assessed using the colorimetric WST-8 cell proliferation assay (see Section 2.4).

5.2.2 The Synergistic Anti-Tumour Activity of fucoidan with ATO in APL-Bearing Mice

An optimization experiment was performed to determine the route and the dose of fucoidan administration. A subcutaneous xenograft tumour was established in 14 female BALB/c nu/nu athymic mice (see Section 2.9.5) and fucoidan was delivered with different doses (high and low) and routes (IP and IV) once tumours was appeared. The best route and dose were selected for the rest of the experiments in chapters 5 and 6 of the thesis.

To examine the anti-tumour effects of fucoidan and its synergy with ATO, 28 mice (n=7/group) were randomly divided into four treatment groups; the control, fucoidan, ATO and fucoidan+ATO. A subcutaneous xenograft tumour was established by injecting NB4 cells in the right flank of mice (see Section 2.9.5). Treatment was commenced following tumour appearance at the following doses: 100 µg/g body weight (b.w.) fucoidan, 2.5 µg/g b.w. ATO or in combination, daily. The control group was treated with IP injection of vehicle (sterile PBS). Mouse weight and tumour volume were measured daily. Once tumour volume reached 1000 mm^3, this time point was considered as the end point and the mice were humanely euthanised with inhalation of CO_2 (see Section 2.9.7). Tumour volume doubling time was calculated for each mouse.

5.3 Results

5.3.1 Fucoidan Increases the Anti-proliferative Activity of ATO in APL Cells *in Vitro*.

The acute promyelocytic leukaemia NB4 cell line was treated with fucoidan plus clinical and sub-clinical doses of ATO for 48 hours, and cell proliferation and apoptosis were evaluated using different assays.

NB4 cell proliferation was significantly decreased when cells were co-treated with fucoidan and ATO, compared to single treatment with either agents (Figures 5.1, 5.2). The decreased proliferation was observed in both low (0.25, 0.5 µM) and clinical doses of ATO (1 µM).

At lowest dose of 0.25 µM ATO, combination of 10 µg/mL fucoidan with ATO resulted in decreased cell proliferation to approximately 80%. Whereas combination of 20 µg/mL of fucoidan with ATO reduced the cell proliferation to near 60%. According to the results obtained, fucoidan at the concentration of 20 µg/mL was used for the rest of the apoptosis experiments.

In the clinic, ATO is only used for treatment of acute promyelocytic leukaemia and not for other sub-types of AML as it is not effective in treatment of other subtypes (286). To examine whether the ATO activity observed was specific to APL cells, the non-APL t(8;21) positive AML Kasumi-1 cell line was similarly treated with ATO plus fucoidan. Neither combined fucoidan+ATO nor single treatment with fucoidan and ATO decreased the proliferation of Kasumi-1 cells and the percentage of viable cells in all groups remained at >80% for up to 96 hours (Figure 5.3).

To investigate whether the reduced cell proliferation in NB4 cells was due to cell death or cell cycle arrest, the DNA content of the NB4 cells treated with fucoidan+ATO was analysed using flow cytometry. The amount of the sub-G0/G1 population, which represents dead cells, significantly increased when cells were treated with fucoidan+ATO compared to ATO alone at both therapeutic and sub-therapeutic doses ($p \leq 0.001$) (Figure 5.4). Table 5.1 displays distribution of all cell cycle phases in NB4 cells.

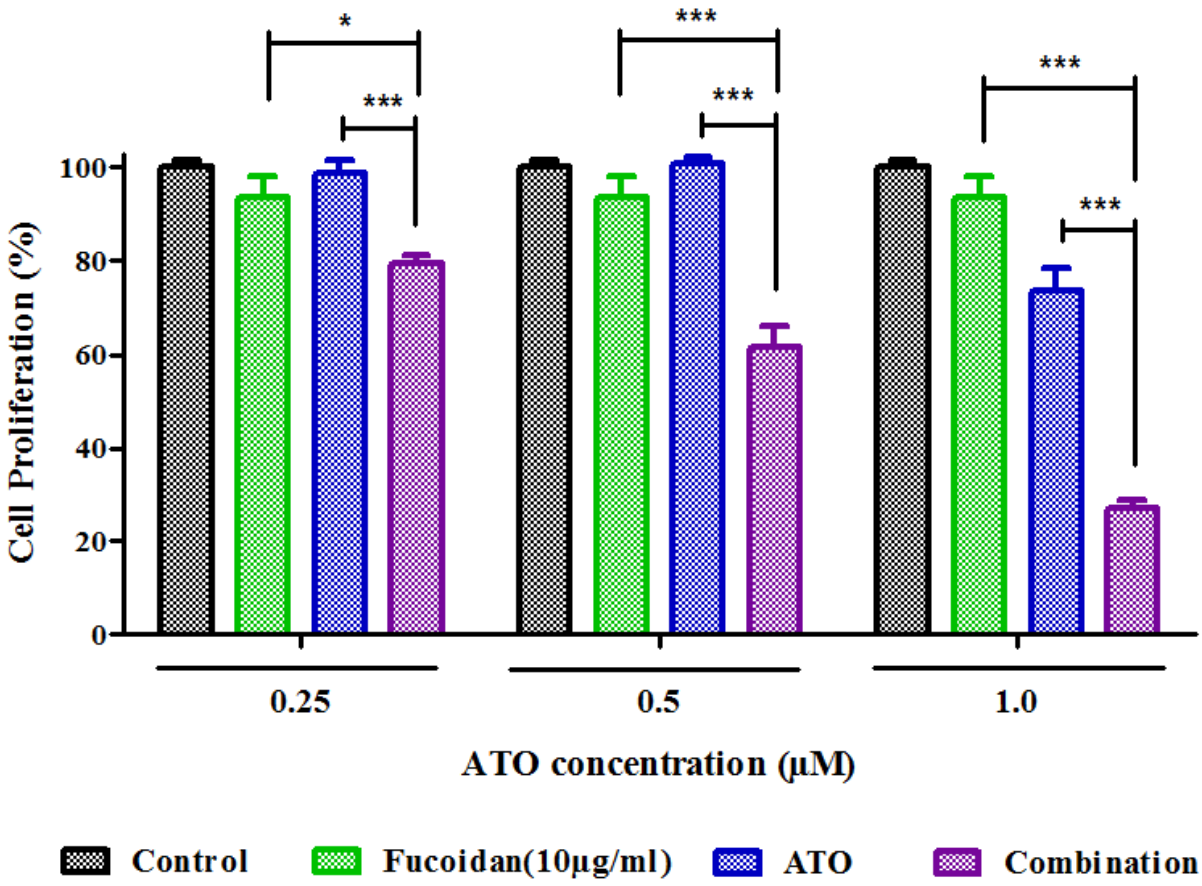


Figure 5.1. Inhibitory effects of ATO plus 10 µg/mL fucoidan on APL cell proliferation. NB4 cells were treated with low to high doses of ATO with or without fucoidan for 48 hours and cell proliferation was evaluated using the WST-8 assay. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by two-way ANOVA, followed by Bonferroni post-test (***:$p \leq 0.001$, *:$p \leq 0.05$).

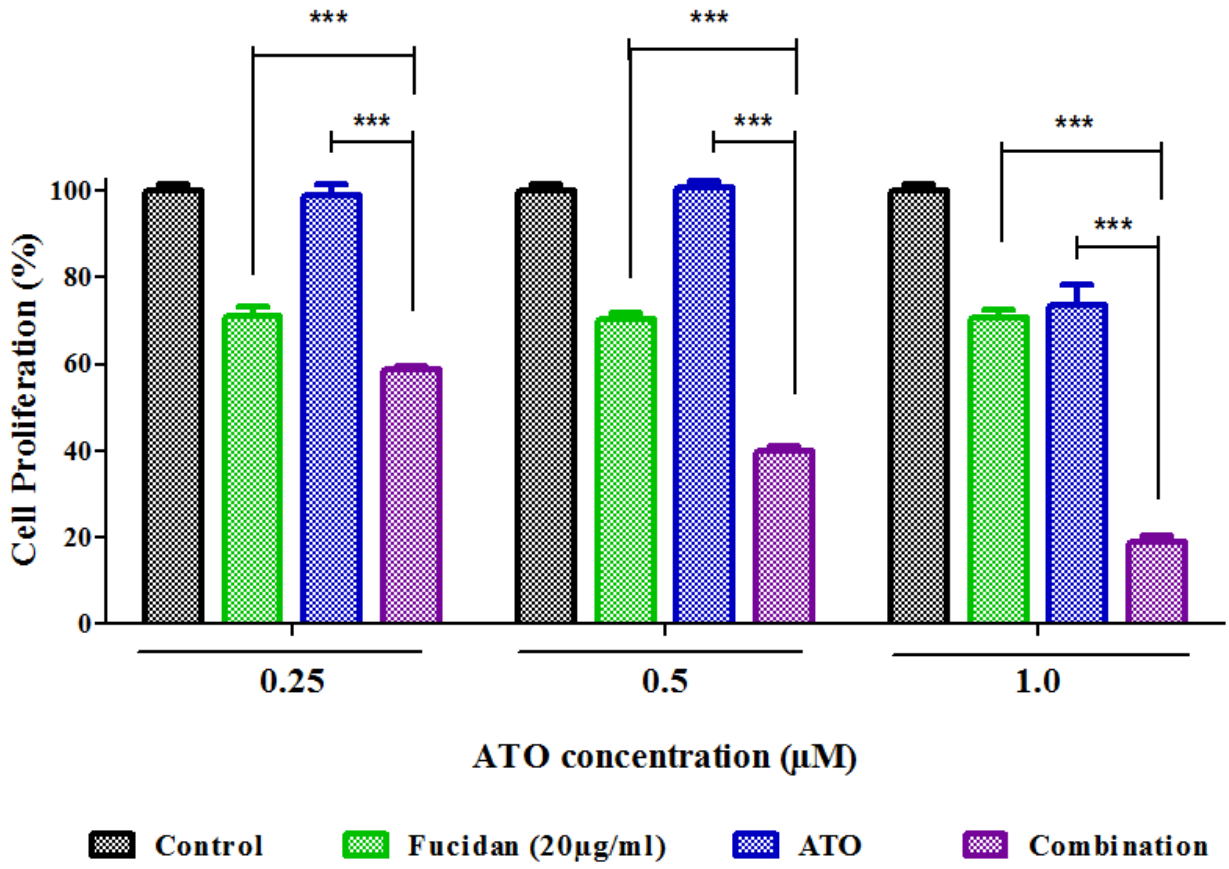


Figure 5.2. Inhibitory effects of ATO plus 20 µg/mL fucoidan on APL cell proliferation. NB4 cells were treated with low to high doses of ATO with or without fucoidan for 48 hours, and cell proliferation was evaluated using the WST-8 assay. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by two-way ANOVA, followed by Bonferroni post-test (***: $p \leq 0.001$).

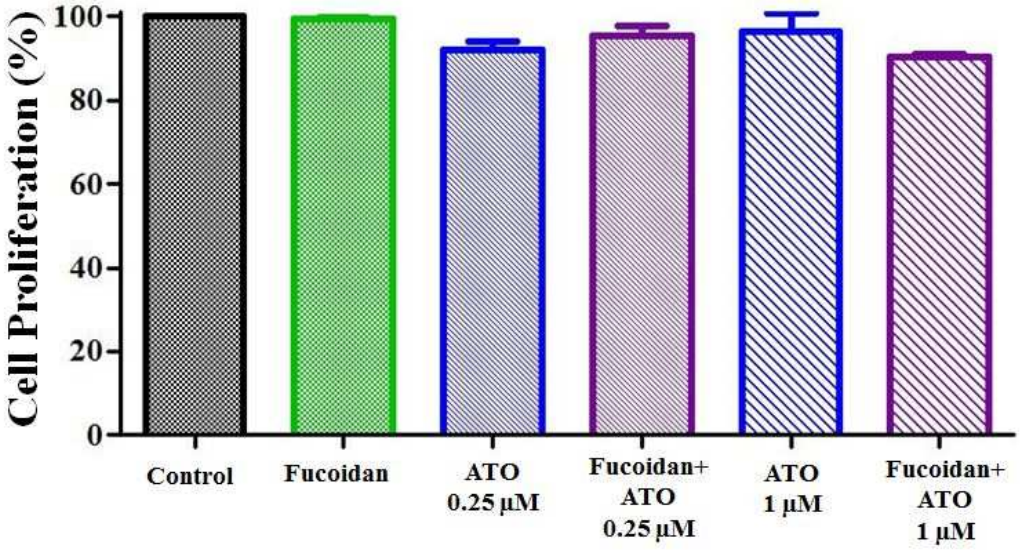


Figure 5.3. Inhibitory effects of ATO combined with fucoidan on non-APL Kasumi-1 cell proliferation. Kasumi-1 cells were treated with low and high doses of ATO with or without 20 µg/mL fucoidan, and cell proliferation was evaluated using WST-8 assay after 96 hours. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test.

Table 5.1. Cell cycle phase distribution. NB4 cells were treated with various doses of ATO with or without fucoidan (20 µg/mL), and DNA content was assessed using flow cytometry after 48 hours. Data represents mean ± SEM of at least three replicates.

		Sub G0/G1	G0/G1	S	Mitosis
ATO 0.25 µM	Control	1.93 ± 0.30	42.46 ± 1.39	22.34 ± 0.06	31.67 ± 1.34
	Fucoidan	18.34 ± 1.75	28.66 ± 1.42	22.38 ± 2.70	29.18 ± 3.38
	ATO	2.73 ± 1.08	45.10 ± 0.54	20.22 ± 0.41	30.39 ± 0.87
	Combination	20.69 ± 1.54	31.30 ± 0.09	25.8 ± 2.68	19.44 ± 2.04
ATO 0.5 µM	Control	1.93 ± 0.30	42.46 ± 1.39	22.34 ± 0.06	31.67 ± 1.34
	Fucoidan	18.34 ± 1.75	28.66 ± 1.42	22.38 ± 2.70	29.18 ± 3.38
	ATO	2.39 ± 1.08	46.10 ± 0.54	20.22 ± 0.41	29.39 ± 0.87
	Combination	25.57 ± 0.86	38.42 ± 1.38	15.20 ± 0.34	18.14 ± 1.66
ATO 1.0 µM	Control	1.93 ± 0.30	42.46 ± 1.39	22.34 ± 0.06	31.67 ± 1.34
	Fucoidan	18.34 ± 1.75	28.66 ± 1.42	22.38 ± 2.70	29.18 ± 3.38
	ATO	15.13 ± 0.40	39.81 ± 3.82	16.56 ± 0.80	25.50 ± 2.11
	Combination	74.51 ± 2.26	7.29 ± 1.36	9.28 ± 2.28	6.76 ± 0.70

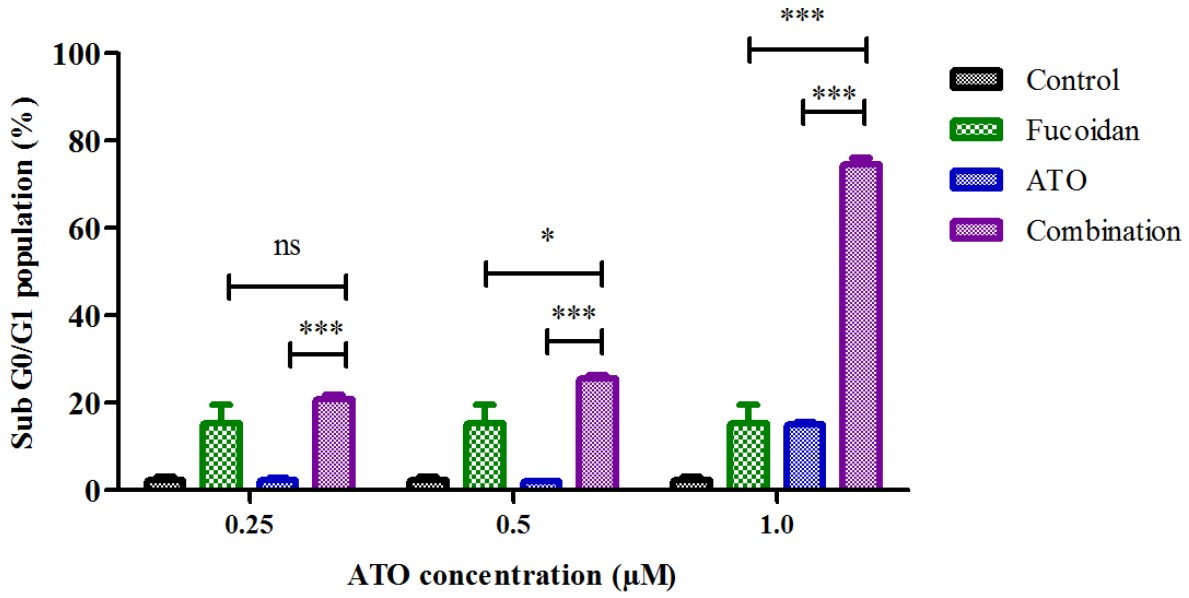


Figure 5.4. Effects of ATO plus fucoidan on accumulation of sub G0/G1 dead cells. NB4 cells were treated with various doses of ATO with or without fucoidan (20 µg/mL) and cell cycle was analysed after 48 hours. Sub G0/G1 population representing the dead cells significantly increased when fucoidan was combined with ATO at both clinical and low doses. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by two-way ANOVA, followed by Bonferroni post-test.
(***: $p \leq 0.001$, *: $p \leq 0.05$)

5.3.2 Fucoidan Synergises with ATO in ATO-Mediated Apoptosis in APL Cells.

To determine if cell death was due to apoptosis, the annexin V/PI assay was employed. Figure 5.5 shows a representative flow cytometry result for each treatment. As shown, combination of fucoidan with ATO significantly enhanced apoptosis in NB4 cells. At low doses of ATO, the percentage of apoptotic cells (annexin V positive cells) increased from approximately 6% (0.25 µM ATO only) to 32% (fucoidan+0.25 µM ATO) and from 5% (0.5 µM ATO only) to 52% (fucoidan+0.5 µM ATO) ($p \leq 0.001$). At the therapeutic dose of 1 µM ATO, the percentage of apoptotic cells increased from approximately 18% (ATO only) to 71% (fucoidan+ATO) ($p \leq 0.001$).

Furthermore, the TUNEL assay was employed to measure the amount of DNA fragmentation; which is one of the main features of apoptosis. Consistent with the annexin V/PI assay data, co-treatment of fucoidan with ATO significantly enhanced apoptosis compared to treatment with ATO only ($p \leq 0.001$). As shown in Figure 5.6, the mean percentage of cells with fragmented DNA increased to 42.5% (fucoidan+0.25 µM ATO), 60% (fucoidan+0.5 µM ATO), and 79% (fucoidan+1 µM ATO) compared to 1.5%, 1% and 12%, respectively, for the same doses of ATO alone.

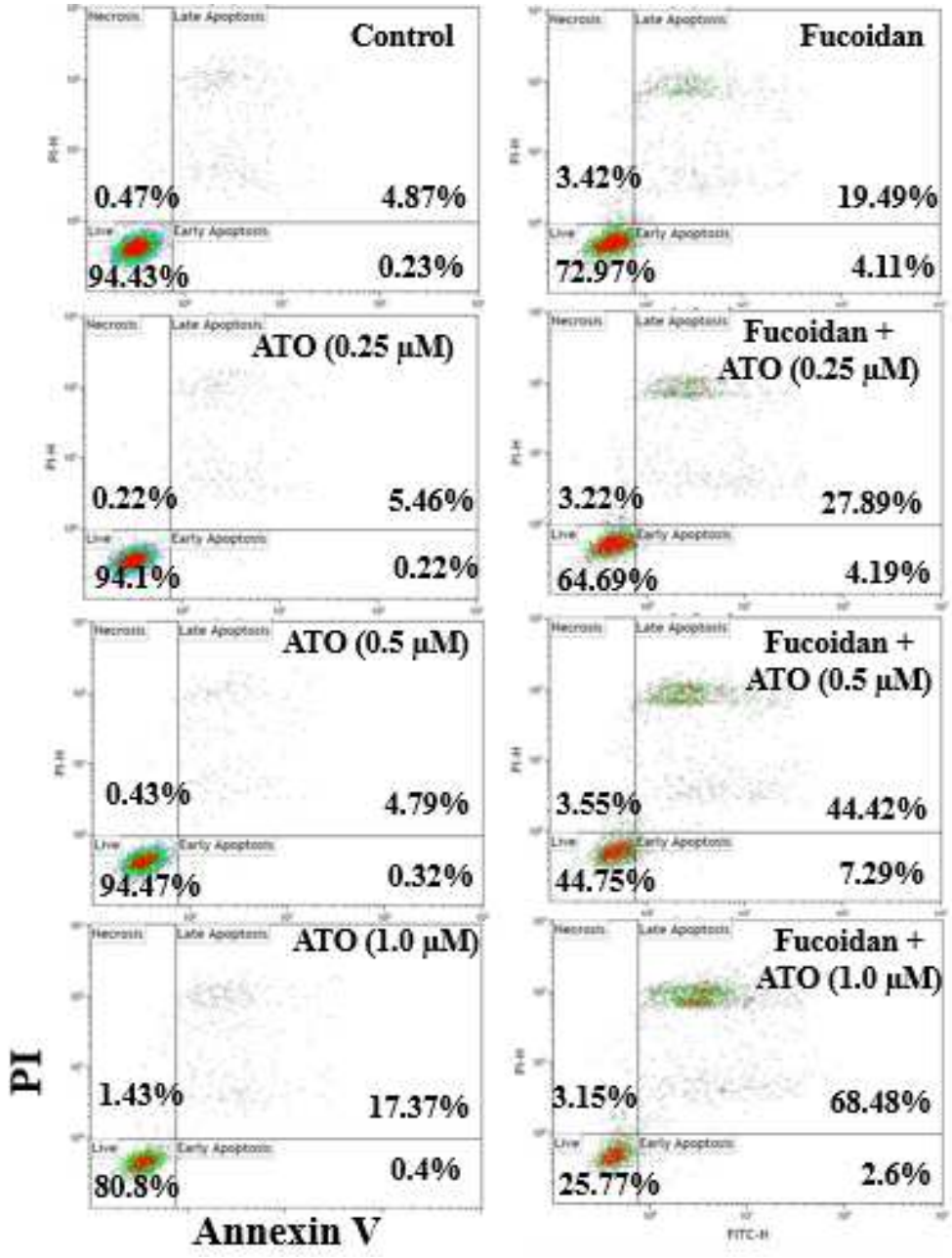


Figure 5.5. Representative annexin V/PI apoptosis assay. NB4 cells were treated with increasing doses of ATO with or without fucoidan (20 µg/mL) and apoptosis was measured using annexin V/PI apoptosis assay. Results represent flow cytometry histograms of one of three replicates.

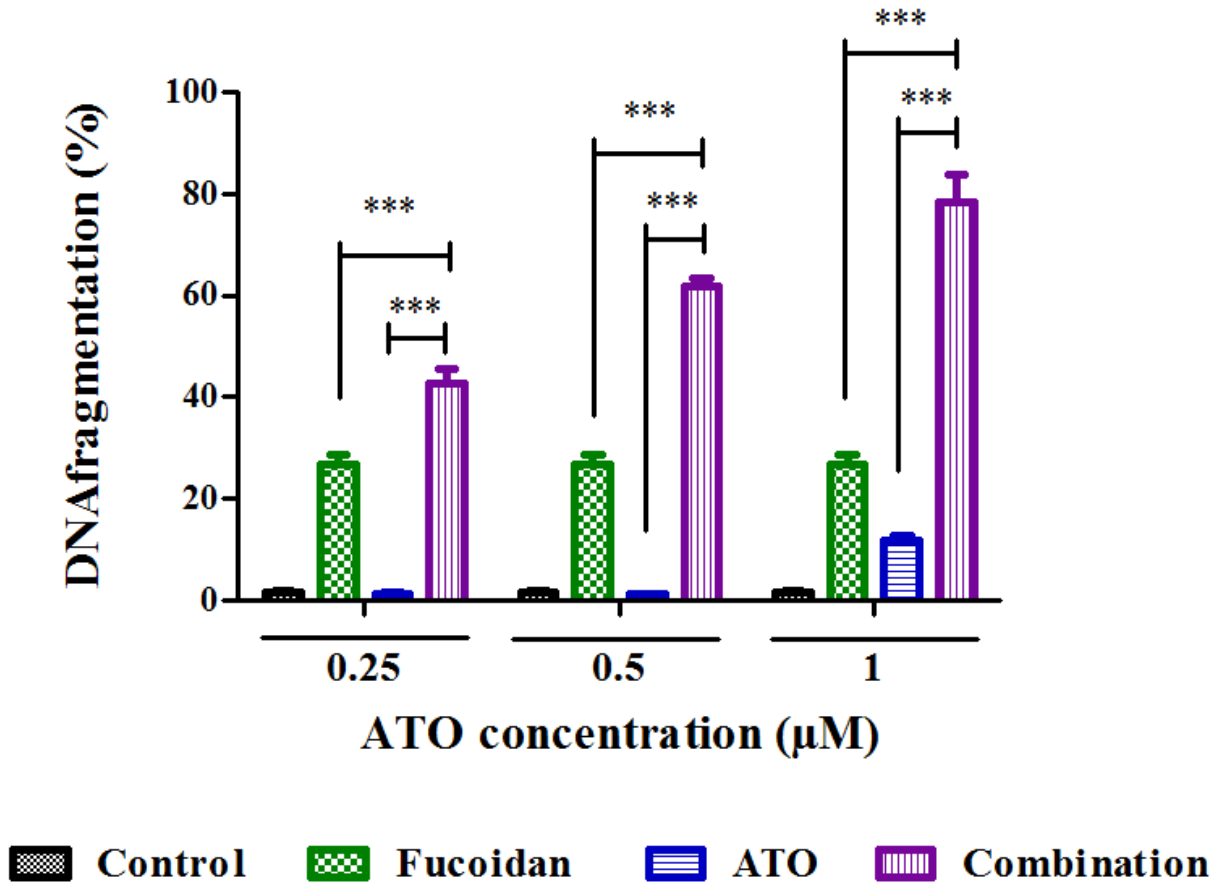


Figure 5.6. DNA fragmentation. The percentage of apoptotic cells with fragmented DNA significantly increased when fucoidan (20 µg/mL) was combined with ATO at both low and high doses. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by two-way ANOVA, followed by Bonferroni post-test (***: $p \leq 0.001$).

5.3.3 High Dose Fucoidan Delayed Tumour Growth

To optimise the route and the dose of fucoidan administration, 14 female mice were divided into three groups according to the dose (low or high) and the route (IP or IV) of fucoidan administration as follow:

1. IP low dose (30 µg/g b.w.)
2. IP high dose (100 µg/g b.w.)
3. IV (6 µg/g b.w.)

A subcutaneous xenograft tumour was established in mice and treatments were applied daily at the mentioned conditions. Out of 14 mice, 5 mice (2 in each IP group and 1 in IV group) did not develop tumours for up to 50 days. These mice were excluded from the experiments and data analysis. The number of mice in different groups was therefore restricted to 9 animals as shown in Figure 5.7.

In the 9 remaining mice, a visible tumour mass (4~10 mm^3) appeared in 8 mice after 8-14 days. The tumour appeared in the last mouse at day 20.

Despite using different techniques, attempts to administer fucoidan through IV injection failed. Due to the small size of nude mice and thin tail blood vessels, fucoidan could not be injected IV for more than 2 or 3 days. As a result, the IV injection was excluded from the experiments and the three mice in IV group were transferred to the IP groups (Figure 5.7).

Figure 5.8 displays the tumour appearance and growth for the optimization assay. The tumour size in all mice in IP-low dose group reached the end point (1000 mm^3) in less than 12 days after the treatment commenced. In the IP-high dose group, the tumour size reached the end point between days 13 to 26. The high dose treatment did not induce any side effects (such as weight loss and/or behavioural changes) in the mice. As a result, the dose of 100 µg/g b.w. and IP injection route were selected for the main experiments in this Chapter and Chapter 6.

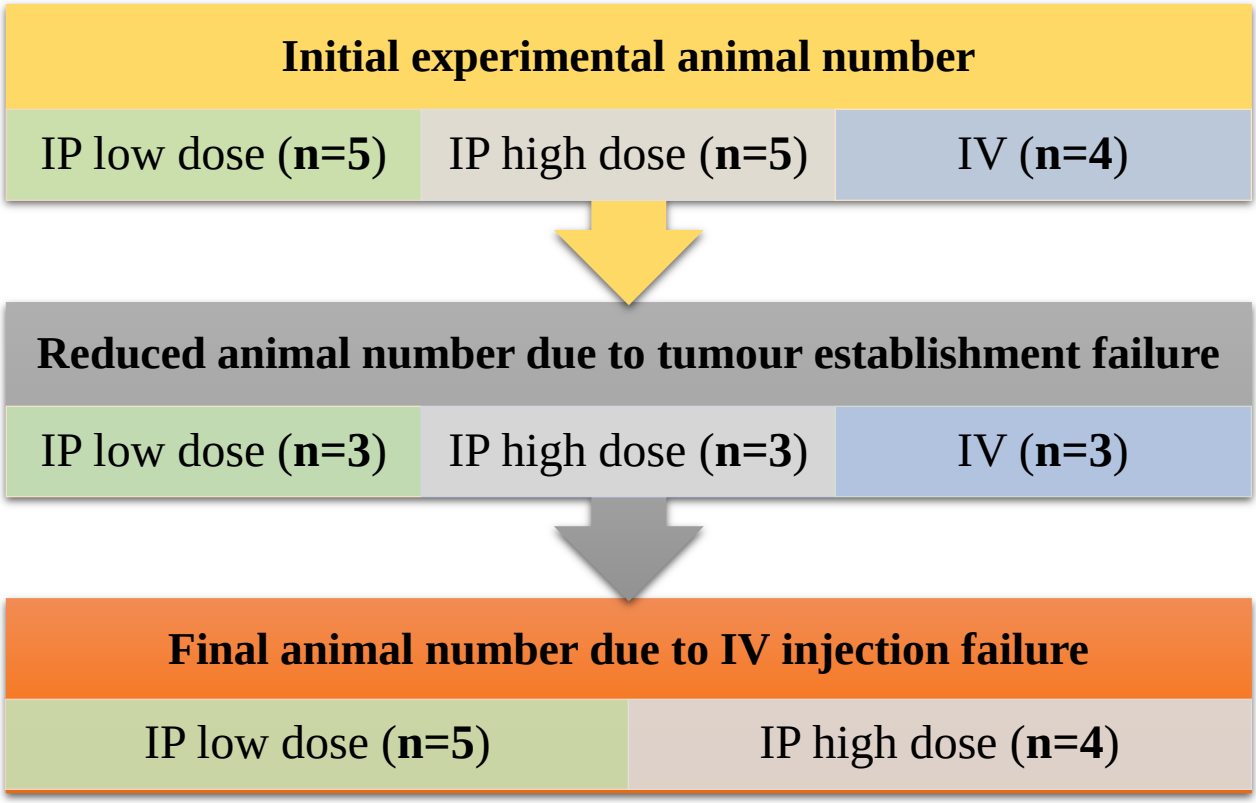


Figure 5.7. Total animal number used in optimization assay. The number of animals decreased due to tumour establishment and IV injection failure.

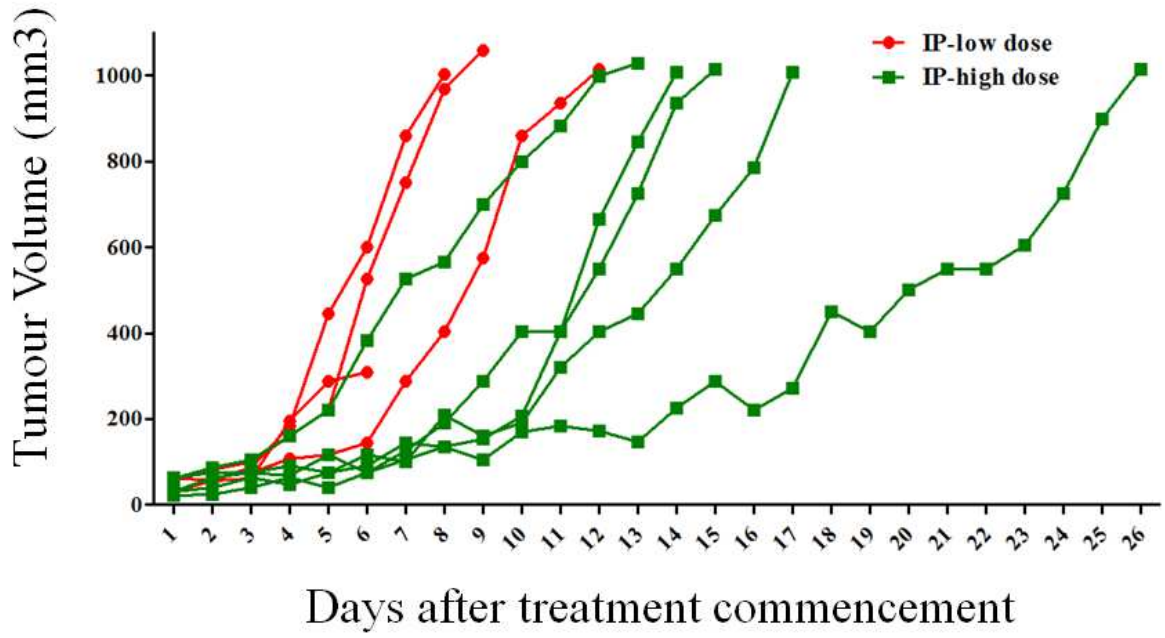


Figure 5.8. Tumour mass growth in mice treated with different doses of fucoidan. Xenograft tumour was established in mice with injection of NB4 cells and treatment with low and high doses of fucoidan (IP) was commenced after tumour appearance. Tumour volume was measured daily. Each graph represents the daily tumour volume of one mouse in high-dose (green) and low dose (red) groups (n=4 in low dose/n=5 in high dose groups).

* Due to tumour rupture, one mouse in the IP-low dose group was sacrificed at day 6 of treatment.

5.3.4. Co-treatment of Fucoidan with ATO Significantly Delayed the Growth of APL in Mice.

To evaluate the possible synergy of ATO and fucoidan *in vivo*, a subcutaneous xenograft APL mass was established in 28 male nude mice (n=7/group) and single or combined treatments of fucoidan and ATO were commenced following tumour appearance. Tumour mass volume was monitored and median survival was identified using the Kaplan-Meier survival analysis.

A visible tumour mass (4~10 mm^3) appeared after 8-14 days in all 28 mice. During the treatment, none of the mice showed any side effects (e.g. weight loss or behavioural changes) to the administered doses. Of the 7 mice treated with fucoidan+ATO, only 2 mice completed the full 28-days treatment. All remaining mice were sacrificed as per animal ethics guidelines prior to 28-days treatment as tumour volume reached 1000 mm^3.

Treatment with "fucoidan only" and "fucoidan+ATO" significantly slowed the increase in tumour mass with the tumours in these animals reaching the maximal end point much later than the control group ($p \leq 0.05$). The median survival of the mice was 14.5, 18.5, 16.5 and 20 days in control, fucoidan alone, ATO alone and combination groups, respectively (Figure 5.9). Although a higher median survival in combination group was observed, the difference between treated groups was not statistically significant.

Tumour aggressiveness was evaluated by tumour volume doubling time which increased in treated groups compared to the control group. The mean tumour volume doubling time was 2.72, 3.70, 3.42 and 3.86 days in control, fucoidan, ATO and combination groups, respectively (Figure 5.10).

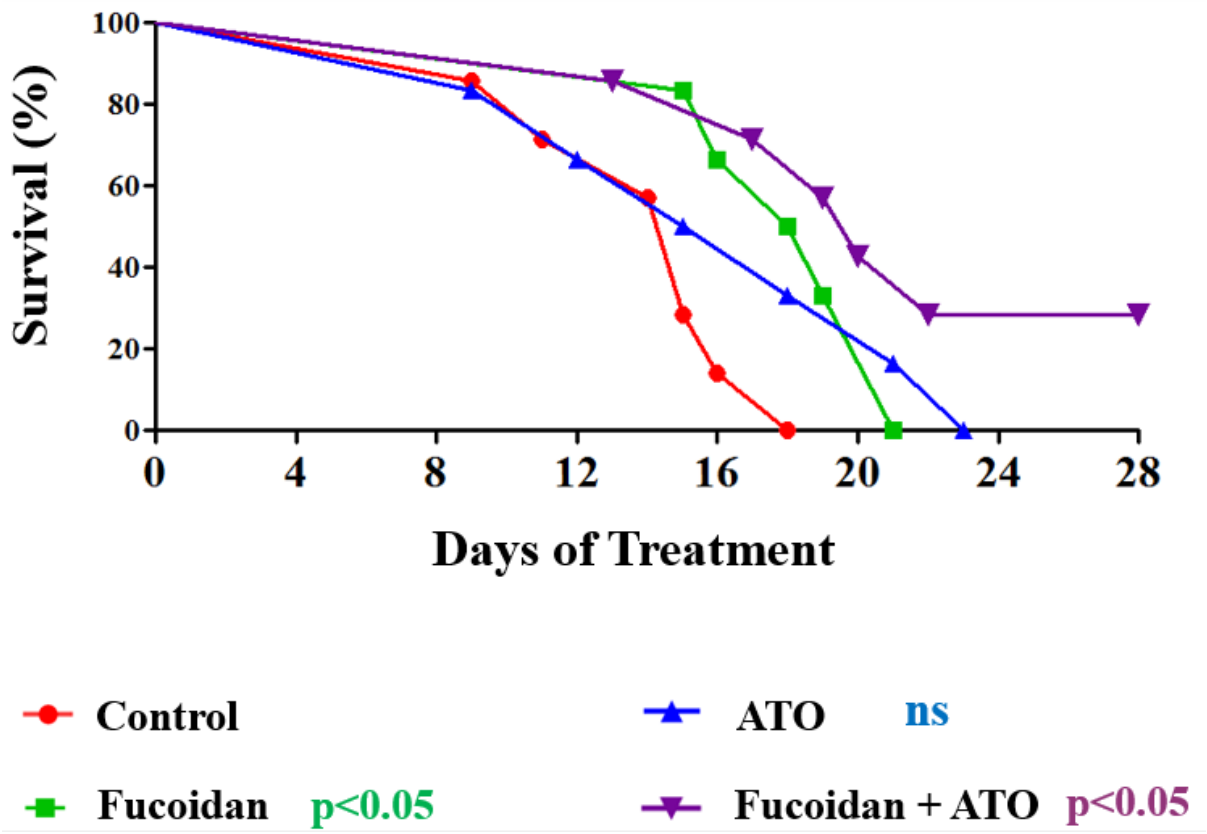


Figure 5.9. The Kaplan-Meier survival analysis over 28 days of treatment. Mice with APL were treated with vehicle (red), fucoidan (green), ATO (blue) and their combination (purple) and their life span was analysed over 28 days of treatment (n=7/ group). *p* values refer to comparison between each treatment group with the control group.

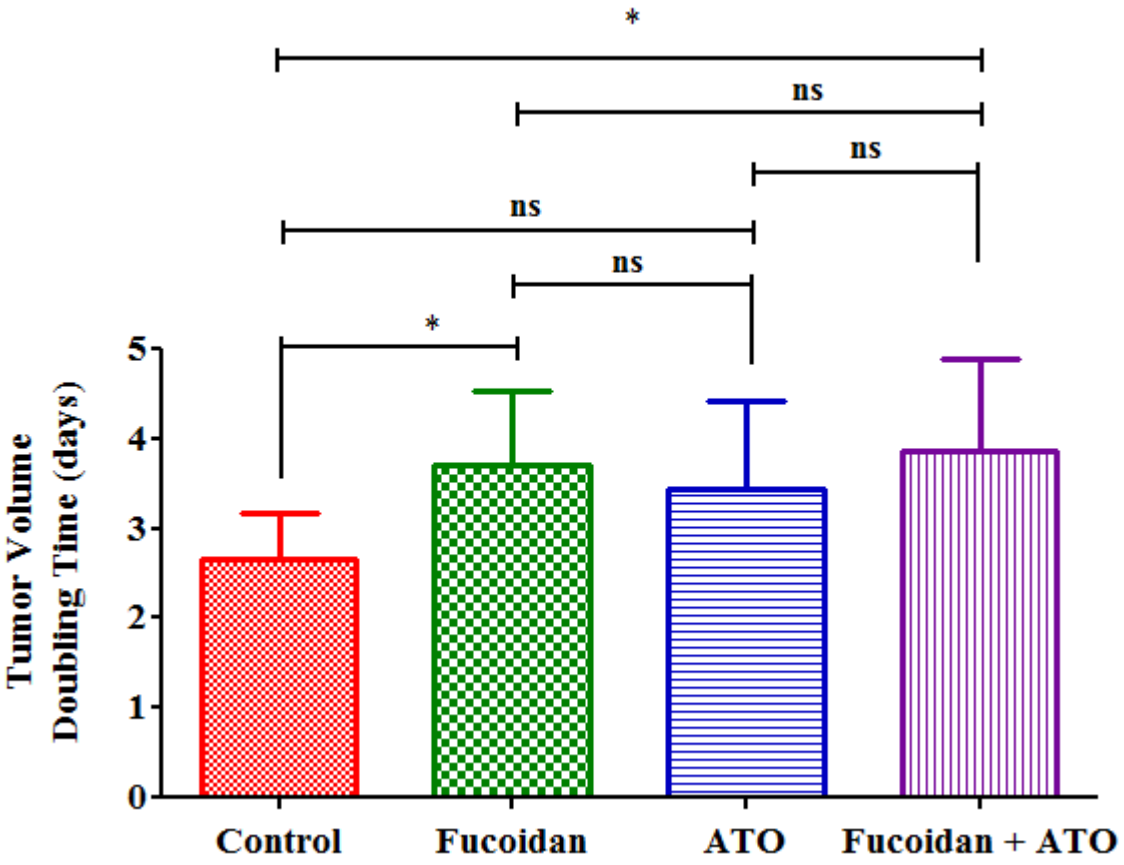


Figure 5.10. Mean tumour volume doubling time. Mice with APL were treated with vehicle (red), fucoidan (green), ATO (blue) and their combination (purple) and their mean tumour volume doubling time was compared to that of the control group (n=7/group). Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test (*: $p \leq 0.05$). Each column shows mean ± SD.

5.4 Discussion

Herein, the synergistic effects of fucoidan with ATO in ATO-induced apoptosis was examined *in vitro* and *in vivo*. Evidence provided here supports the hypothesis that combining fucoidan with ATO enhanced apoptosis at both clinically-used and low doses of ATO, *in vitro*. In addition, enhanced survival and delayed tumour growth were observed in APL-bearing mice when treated with combined fucoidan and ATO. This is the first study to examine the potential therapeutic activity of fucoidan in leukaemia *in vivo*.

5.4.1 Synergistic Effect of Fucoidan with ATO *in Vitro*

In the last decade, combined treatment of natural components with chemotherapeutic agents has gained considerable attention. The potential synergistic activities of these agents could be exploited to reduce the toxicity of chemotherapy by achieving the same (or even higher) efficacy at lower doses. ATO was first used for APL treatment in the early 1990s and improved the complete remission rate in APL patients (280). Further studies and clinical trials showed that it induced a sustained molecular remission when it was used alone in relapsed patients (37, 38). However, the currently used clinical doses of ATO has been accompanied with morbidities.

Few studies have examined the combinatory activity of natural derived compounds with ATO in treatment of APL. Lu *et al*. reported that combination of a crude methanolic extract of *Mucuna macrocarpa* with ATO suppressed proliferation of APL cell line HL60 and increased apoptosis through accumulation ROS (287). Fan *et al*. also showed that addition of resveratrol, a stilbene enriched in red wine, to ATO significantly enhanced the anti-cancer effect of ATO in APL cell line NB4 (288). In a recent investigation, the synergistic activity of Icarrin, a natural flavonoid derived from several plants, with ATO was evaluated in NB4 cell apoptosis (289). The combination of these extracts resulted in continuous accumulation of ROS and excessive cellular oxidative stress.

As mentioned before, the clinical doses of 1 µM of ATO can cause side effects in patients. The aforementioned studies have used high doses of ATO; from 2 to 12 µM, which are considerably higher than clinical doses. Here, it was postulated that lower concentrations of ATO could attenuate its undesirable side effects. Therefore APL cells were co-treated with lower doses of

ATO and compared the outcomes with therapeutic doses. Addition of fucoidan to ATO significantly increased the efficacy of ATO in induction of apoptosis in NB4 cells both at low and clinical doses of ATO.

It should be noted that due to the cytotoxic effect of fucoidan in NB4 cells, ultra-low concentrations of fucoidan were used in this chapter than previously used in Chapter 3 (10-20 µg/mL vs 50-100 µg/mL). The lower doses of fucoidan allowed us to identify the effect of fucoidan on ATO-induced apoptosis, and yet even the combination of this low concentration of fucoidan with ATO decreased cell proliferation to less than 20% compared to around 80% in ATO-alone treated cells.

It is well known that ATO induces apoptosis at higher doses whereas it induces myeloid differentiation at lower doses. Here, as expected, high dose of ATO (1 µM) increased the sub-G0/G1 dead cell population in the cell cycle assay. Consistent with the literature, lower doses of ATO (0.25 and 0.5 1 µM) did not induce apoptosis in APL cells and did not increase the sub G0/G1 dead cell population. However, they induced G0/G1 arrest with 42% cells in control compared to near 46% in ATO-treated cells. The slight G0/G1 arrest within 48 hours could be associated with initiation of differentiation in APL cells in response to low dose ATO. This effect will be discussed in detail in Chapter 6.

AKT, a serine/threonine kinase, is a key molecule in PI3K/AKT signaling pathway which inhibits apoptosis and is reported to be frequently upregulated in various tumours (290). The results of Chapter 3 of this thesis together with other studies have shown that fucoidan down-regulates the activity of AKT (163, 180). Further, there are several reports that show that ATO triggers apoptosis via inhibition of AKT (291, 292). Evidence indicates that fucoidan and ATO trigger the same pathway and therefore it could be postulated that double inhibition of AKT may explain the observed synergistic activity.

Evidence shows that apoptosis mediated by ATO is caspase independent (293, 294). It induces apoptosis through down-regulation of anti-apoptotic molecule Bcl-2 expression, induction of accumulation of reactive oxygen species (ROS) and disruption of mitochondrial transmembrane potentials ($\Delta\Psi m$), eventually resulting in increased cleavage of PARP-1 and subsequent cell death. PARP-1 is a nuclear enzyme which modifies various nuclear proteins involved in different cellular processes. PARP-1 has a key role in DNA repair and cell recovery

from DNA damage through maintenance of genomic integrity and its inactivation is highly associated with apoptosis (295). Cleavage of PARP-1 is one of the main mechanisms triggered by a wide range of anti-cancer agents. Similar to ATO, in Chapter 3 it was shown that fucoidan significantly increased the cleavage of PARP-1, however through a caspase dependent process. Thereby, although fucoidan and ATO recruits different pathways, both agents eventually induce cleavage of PARP-1 (285). This may explain the severe induced DNA fragmentation when ATO was combined with fucoidan (80%) compared to when it was used alone (about 15%).

5.4.2 Therapeutic Potential of Fucoidan in APL-Bearing Mice

Next, the therapeutic effects of fucoidan on APL-bearing mice was examined and its synergy with current APL therapies was evaluated *in vivo*.

In previous *in vivo* studies examining the therapeutic effect of fucoidan, the range of utilised doses mostly resides between 5 to 500 µg/g b.w. To select the more effective dose, Lee and colleagues treated mice with a range of 0, 10, 50, 100, 200 and 400 µg/g b.w. fucoidan 24 hrs and 1 hr before irradiating the mice. The mice injected with 100 µg/g b.w. fucoidan showed the best survival rate at 30 days post-irradiation (296). Here, mice were treated with 30 µg/g b.w. and 100 µg/g b.w. and it was found that the mice treated with high doses of fucoidan had delayed tumour growth compared to the low-dose treated group, suggesting the therapeutic potential of fucoidan in treatment of mice bearing APL.

Similar to Chapter 4 (see Sections 4.3.1 and 4.4), the utilised mouse model had some limitations and female mice were less susceptible to developing tumours. Thereby, subsequent experiments were conducted on male mice.

5.4.3 Synergistic Effect of Fucoidan with ATO *in Vivo*

Despite many *in vitro* studies investigating the synergistic activity of fucoidan with anti-cancer drugs, *in vivo* studies have been limited. Alekseyenko *et al*. reported that in C57Bl/6 mice fucoidan significantly potentiated the anti-metastatic, but not anti-tumour activity of the chemotherapeutic agent cyclophosphamide (284). In a clinical trial, fucoidan similarly did not

affect the efficacy of standard chemotherapeutic agents in patients with colorectal cancer, but reduced the toxicities of chemotherapy (193). The previous reported experiments in this Chapter and Chapter 4 provided evidence that fucoidan was effective in delaying tumour growth *in vivo*. These data led to examining the combinatory therapeutic effects of fucoidan with low doses of ATO in APL-bearing mice.

Unlike other AML subtypes, early death is the primary cause of treatment failure in patients with APL, mainly due to the toxicity of therapeutic agents themselves (297). This toxicity has been reported in *in vivo* studies as well. In an *in vivo* study, Jing and colleagues treated APL-bearing mice with 8 µg/g b.w. of ATO and reported toxicity with weight loss and early-treatment associated deaths in mice (40). Lallemand-Breitenbach *et al*. also reported that 10 µg/g b.w. of ATO was toxic and caused many early deaths due to hepatic toxicity and widespread pulmonary edema (41). Hence, they used a reduced dose of 5 µg/g b.w. ATO in mice bearing APL. In this thesis, with the assumption that the lower concentrations could attenuate the side effects of these drugs, the dose was reduced even further (2.5 µg/g b.w. ATO) and no toxicity was observed when it was used alone or in combination with fucoidan. Treatment with fucoidan plus low dose ATO increased the tumour volume doubling time compared to the control ($p=0.0175$) and ATO alone ($p=0.56$) groups. The survival rate in the combination group was the highest between all treated groups, although it was only significant compared to the control group but not the single fucoidan and single ATO treated groups.

ATO alone did not have a significant impact on mouse survival and the tumour doubling time compared to the control group. The utilised low dose may explain the non-significant anti-tumour effects of ATO alone in these investigations. More importantly, in contrast with studies by Jin *et al* and Lallemand-Breitenbach *et al*., no toxicity was observed when this low dose of ATO was used alone or in combination with fucoidan. Even though very low doses were used, the efficacy of combined treatment was still higher than that of standard APL treatment alone.

In conclusion, the data presented in this chapter suggest that the usage of fucoidan as a supplementary agent to the APL standard treatment ATO may represent a promising new strategy for management of APL.

CHAPTER SIX

Investigation of Possible Synergistic Effects of Fucoidan and ATRA in APL Treatment *in Vitro* and *in Vivo*

6.1 Introduction

The PML/RARα gene product of the chromosomal t(15;17) translocation blocks the differentiation of malignant promyelocytes in APL (22). In the late 1970s, the hypothesis of inducing differentiation rather than apoptosis in tumour cells was proposed and drugs with differentiation-inducing property were developed (26). ATRA was the first successful differentiation-based therapy used in patients with APL. However it could only induce transient remission (298). Further pre-clinical studies showed a dramatic synergy between ATRA and arsenic trioxide in inducing differentiation and clearing the APL cells in animal models (41). Today, ATO+ATRA is a standard therapeutic strategy which provides an effective differentiation-based therapy, potentially avoiding use of anthracycline-based chemotherapy.

Retinoic acid receptor alpha (RARα) is a ligand-dependent transcription factor that regulates the expression of a number of genes involved in cell differentiation and proliferation. In normal promyelocytes, RARα binds to the promoter region of target genes and recruits various molecules such as nuclear co-repressor proteins (N-coR). These molecules form complexes with histone deacetylase enzymes (HDACs), leading to transcriptional repression and differentiation block (28). ATRA is a retinoid derivative which is present physiologically in human plasma. At normal physiological concentrations (10^{-9} to 10^{-8} M), conformational changes occurs in the RARα, leading to dissociation of the co-repressors/HDAC complex and re-expression of differentiation-related proteins (Figure 6.1A). In malignant promyelocytes the fusion protein PML-RARα is attached to the gene promoters and at physiological concentrations, ATRA cannot dissociate the co-repressors/HDAC complex, causing the persistence of transcriptional repression and differentiation arrest (28, 283) (Figure 6.1B). At higher pharmacological doses of ATRA, within range of 10^{-7} to 10^{-6} M, the co-receptor proteins are removed, relieving transcriptional repression complexes leading to terminal differentiation of abnormal promyelocytes (Figure 6.1C). This is known as the "reactivation" model. The terminally differentiated mature cells arising from neoplastic clones eventually undergo apoptosis (299).

An additional model for the ATRA-mediated differentiation has been proposed. ATRA has been shown to induce the catabolism of the PML-RARα fusion protein through binding to and targeting the RARα moiety for ubiquitin/proteasome pathway. The degradation of the fusion protein can thus promote differentiation in abnormal promyelocytes (300). This is known as the "de-repression" model. Figure 6.2 represents the two proposed models.

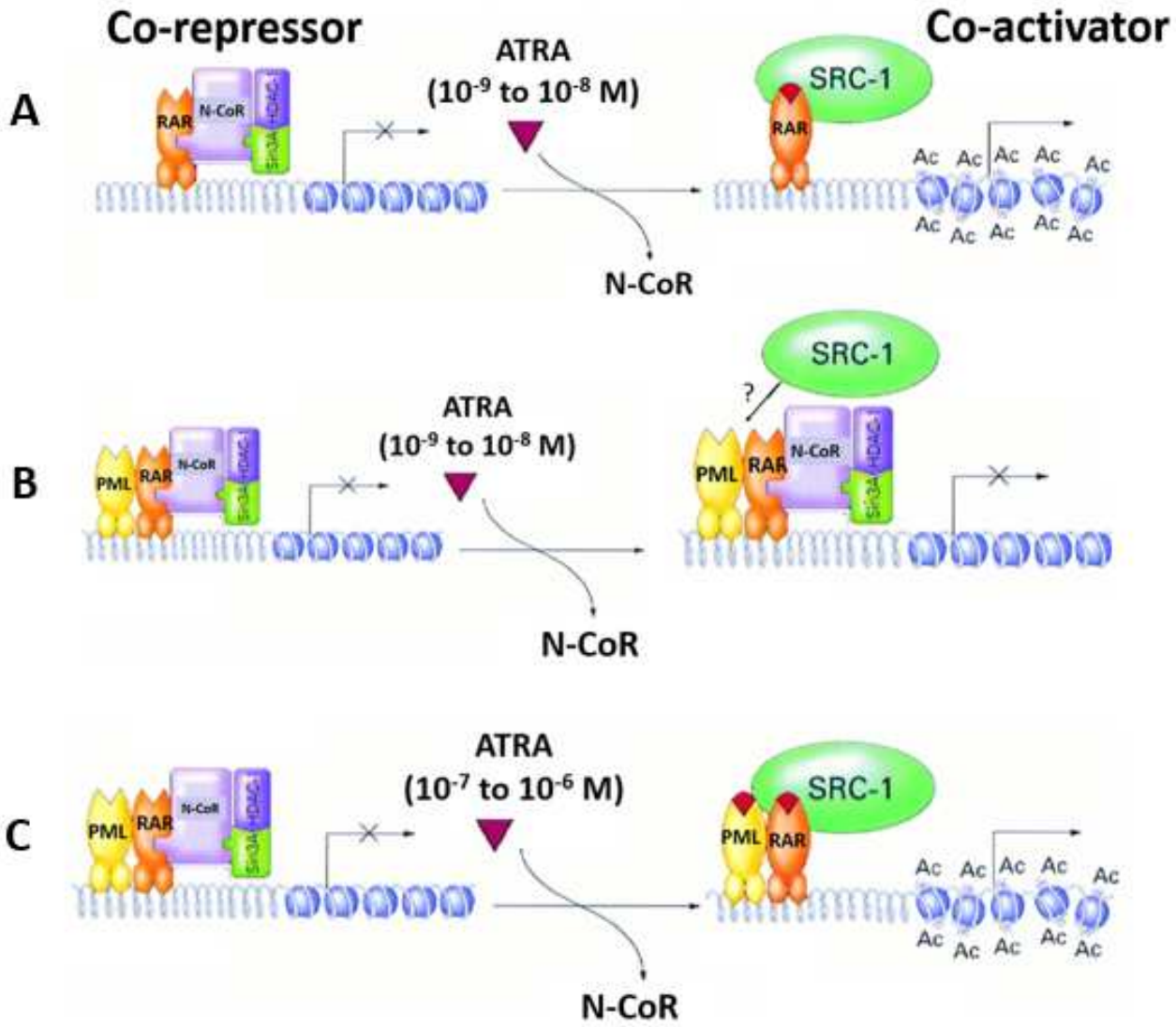


Figure 6.1. Proposed mechanisms of actions of ATRA on nuclear co-repressor complex and PML-RARα fusion protein in APL. In the absence of ATRA, RARα associate with the co-repressor/HDAC complex, which induces reorganization of chromatin structure into a repressed state that is inaccessible to basal transcription factors. **A)** In the normal promyelocyte, physiological doses of ATRA binds to RARα, dissociate the co-repressor complex and causes association of a co-activator such as SRC-1 (301) allowing the access of transcription factors and transcription activation. **B)** In APL, physiological doses of ATRA is not sufficient to dissociate PML-RARα fusion protein from the co-repressor complex. **C)** In APL, pharmacological doses of ATRA are required to dissociate the PML-RARα from co-repressor complex and activate transcription and differentiation (Modified image from (302)).

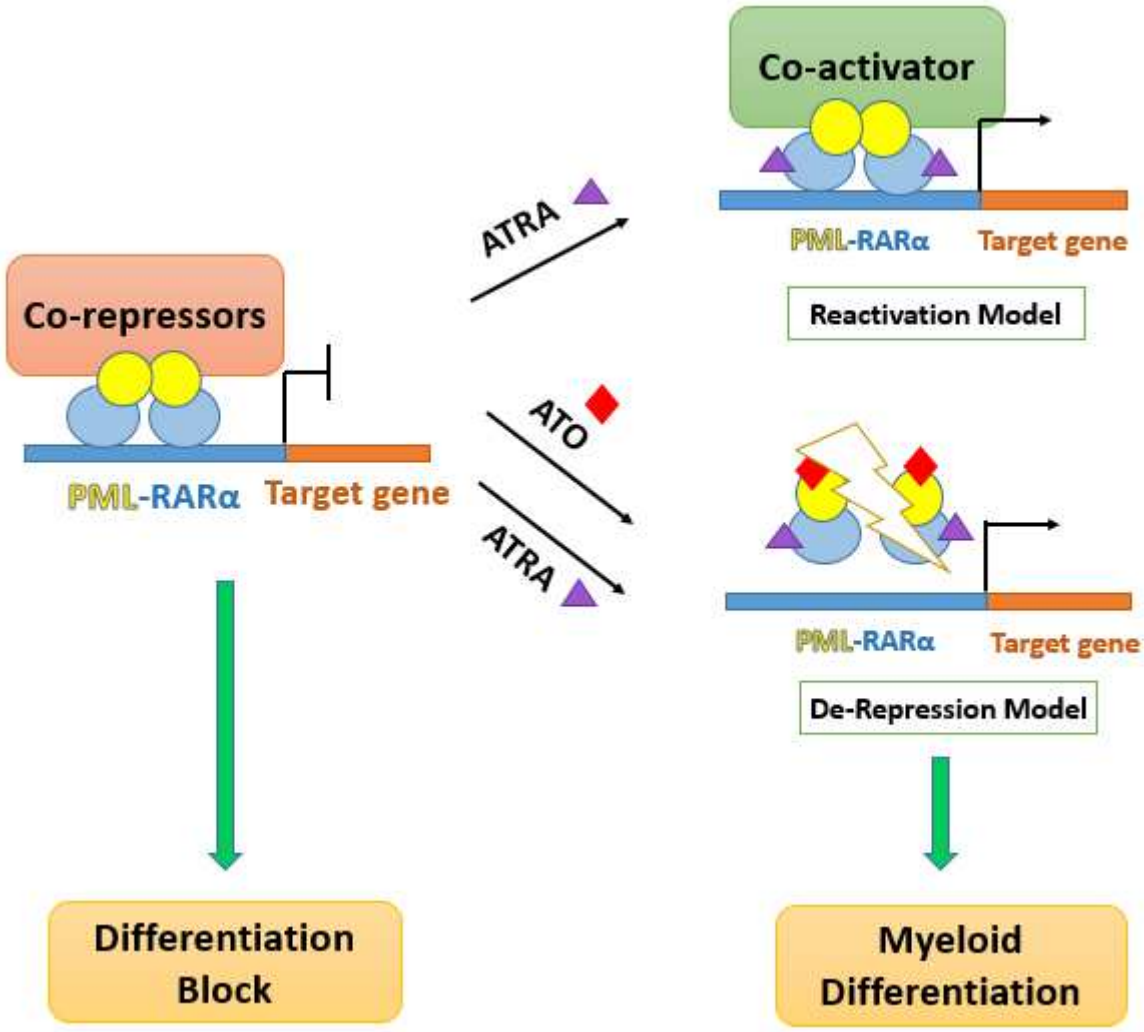


Figure 6.2. Reactivation and de-repression models for the role of ATRA and ATO in induction of differentiation in APL cells. Two current models of ATRA action on differentiation through gene transcription re-activation (top) and action of ATRA and ATO on degradation of PML-RAR or de-repression (bottom) ((Modified image from (298)).

As discussed in Chapter 5, ATO induces both partial differentiation and apoptosis in leukaemic promyelocytes. Similar to ATRA, ATO induces the degradation of the PML-RARα fusion protein, but through targeting the PML moiety (303). Promyelocytic leukaemia (PML) protein is a tumour suppressor protein involved in the regulation of apoptosis, and therefore its degradation upon ATO exposure induces apoptosis in malignant promyelocytes. In terms of the ATO-mediated partial differentiation, it does not change the nuclear receptor signalling and therefore the transcription "reactivation" model cannot account for its action. As mentioned above, ATO degrades PML-RAR fusion protein. Thus, it has been proposed that ATO induces differentiation through abrogation of PML/RARα-mediated gene repression (the "de-repression" model) (304) (Figure 6.2).

It is notable that the advantage of ATRA plus ATO therapy is reduced toxicity compared to standard ATRA plus anthracycline-based chemotherapy. However, despite the enormous improvement in APL treatment, the use of ATRA plus ATO is not free of side effects. Several clinical trials have reported that the prolonged administration of high doses of ATRA results in resistance (32). Moreover, this combination can cause differentiation syndrome; a potentially fatal complication which occurs in approximately 25% of APL patients (281). It has also been reported that the efficiency of myeloid cell differentiation could be diminished due to down-regulation of RARα in the duration of the treatment (305). Therefore, it is of interest to develop complementary treatment strategies which increase the sensitivity of myeloid cells to the action of ATRA+ATO therapy. New adjuvants permitting lower doses of ATRA and ATO to be used could also reduce the toxicity of current treatment regimens in some patients.

As discussed in the previous chapters, fucoidan induces strong anti-tumour activities in various tumour cells and has been shown to be non-toxic when consumed. In recent years, some studies have investigated the synergistic effects of fucoidan with standard anti-cancer agents. Lv *et al.* showed that fucoidan reduced the migration and invasion of multiple myeloma (MM) cells treated with the chemotherapy drug cytarabine (194). Dietary fucoidan synergistically reduced cell growth in the OE33 cell line when it was combined with lapatinib, a targeted therapy that acts as a tyrosine kinase inhibitor in advanced HER2-positive breast cancer cells (197). In Chapter 5 of this thesis, the study of combined effects of fucoidan and the APL standard treatment ATO showed that fucoidan synergistically increased apoptosis induced by ATO in APL cells *in vitro*.

Here, it is hypothesised that fucoidan synergistically enhances the effects of current APL therapeutic regimens, ATRA and ATO, in APL cell differentiation. It has been well documented that exposure of APL cells to ATRA at a concentration of 1 μM results in 95% of cells differentiating accompanied by loss of their capacity to proliferate (306). ATO however induces APL cell partial differentiation at lower doses of 0.1 to 0.5 μM. Since the clinical doses of 1 μM of ATRA and ATO can cause side effects in patients, it is hypothesised that the reduced concentrations could reduce the side effects of these drugs. Therefore, the synergistic effects of fucoidan with ATRA+ATO were investigated in APL cell myeloid differentiation at sub-pharmacological doses *in vitro* and *in vivo*.

6.2 Experimental Design

6.2.1 The Synergistic Effect of Fucoidan with Low Doses of ATRA+ATO in APL Cell Differentiation *in vitro*

The chromosomal t(15;17) translocation positive human APL cell line NB4 was treated with ultra-low dose (5 µg/mL) of fucoidan from *F. vesiculosus* alone, 0.5 µM ATRA alone, 0.5 µM ATO alone or in combinations for 120 hours (see Section 2.2). Monitoring of NB4 cell differentiation was performed by quantitation of the cell surface myeloid differentiation marker CD11b and cell cycle assay by flow cytometry (see Section 2.3).

6.2.2 The synergistic Anti-Tumour Activity of fucoidan with ATRA in APL-Bearing Mice

To examine the anti-tumour activity of fucoidan and its synergy with ATRA, 32 mice (n=8/group) were randomly divided into four treatment groups; the control, fucoidan, ATRA and fucoidan+ATRA. A subcutaneous xenograft tumour was established by injecting NB4 cells in the right flank of mice (see Section 2.9.5). Treatment was commenced by IP injection following tumour appearance at the following doses: 100 µg/g b.w. fucoidan (daily), 1.5 µg/g b.w. ATRA (three times a week) or in combination. The control group was treated with sterile vehicle (see Section 2.9.4). Mouse weight and tumour volume were measured daily. Once tumour volume reached 1000 mm^3, this time point was considered as the end point and the mice were humanely euthanised with inhalation of CO_2 (see Section 2.9.7). Tumour volume doubling time was calculated for each mouse. At the end point, tumour mass was removed and tumour cell differentiation was analysed by CD11b expression.

6.3 Results

6.3.1 Fucoidan Enhances ATRA-Induced Differentiation in APL Cells.

Increasing surface expression of CD11b is a marker of myeloid cells undergoing differentiation (307). To identify the expression level of CD11b, cells were incubated with anti-CD11b antibody and isotype-matched negative control and analysed by flow cytometry. The intensity of CD11b expression was determined using mean fluorescent intensity (MFI). This was first optimised using untreated NB4 cells (Figure 6.3). As shown in Figure 6.3, the signals lower than 10^1 contributed to "neg/low" CD11b expression (non-differentiated cells) and signals higher than 10^1 contributed to "high" CD11b expression (differentiated cells).

Myeloid differentiation was assessed by quantitating CD11b expression following treatment with low doses of ATRA and ATO for 48, 72, 96 and 120 hours. The non-differentiated or low-differentiated $CD11b^{neg/low}$ cells were excluded from the analysis. The percentage of $CD11b^{high}$ cells was quantified as differentiated cells. As shown in Figure 6.4 and 6.5, the CD11b expression increased in ATRA-treated cells from 48 hours and reached a maximum of 83.5% after 120 hours. In contrast, the differentiation induced by ATO was delayed; with increases after 96 hours and reached 20% after 120 hours compared to 2.66% at time zero. For the rest of the experiments, 120 hours was selected for incubation time for the differentiation assay.

To determine the effect of combining fucoidan with ATRA and ATO on APL cell differentiation, NB4 cells were treated with fucoidan, sub-clinical doses of ATRA and ATO, or in combination for 120 hours and differentiation was measured by expression of CD11b as described above. Figure 6.6 demonstrates representative flow cytometry histograms in cells treated with single or combination of different compounds as indicated. As shown, 0.5 µM ATRA increased the differentiated cells from approximately 6% to 83%, while addition of fucoidan to ATRA increased the differentiated cells to 94%. Low-dose ATO similarly increased the differentiated cells from 6% to 14% while ATO+fucoidan enhanced the percentage of differentiated cells to 20.2%. Finally, APL cells treated with all three reagents in combination resulted in a 99% $CD11b^{high}$ cells after the 5-day incubation period, compared to 87% when cells were co-treated with ATRA+ATO.

Taken together, combined treatment of fucoidan with ATRA and ATO significantly increased myeloid differentiation in APL cells after 120 hours (Figure 6.7). Fucoidan alone did not affect myeloid differentiation in APL cells *in vitro* compared to untreated control (Figure 6.7).

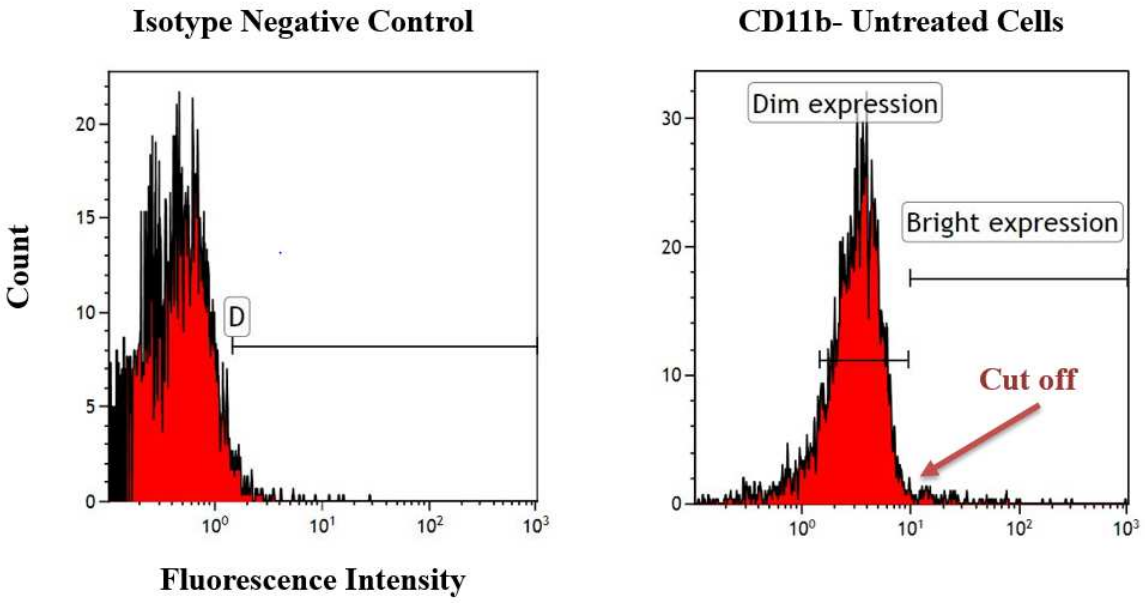
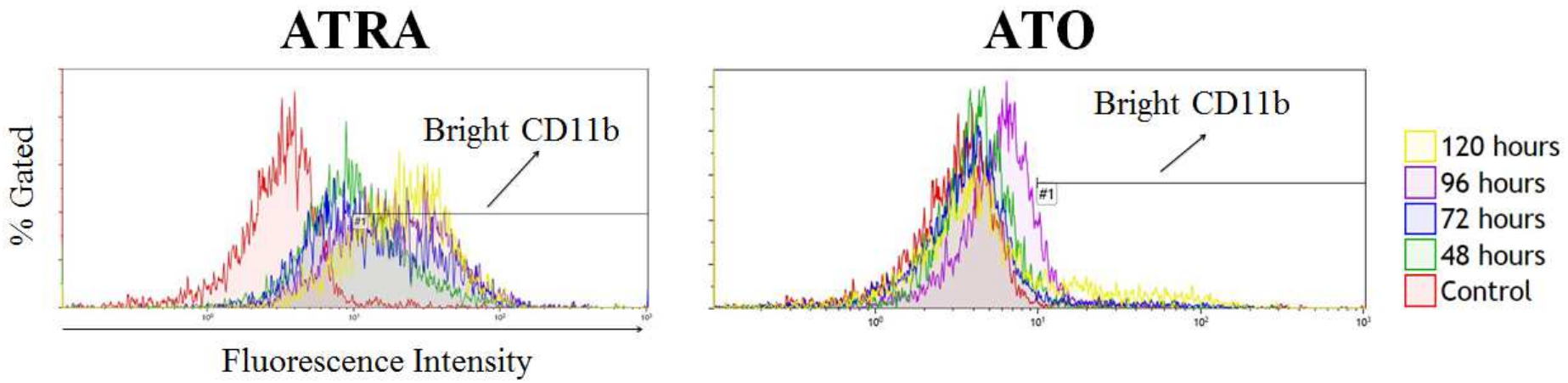


Figure 6.3. Cell differentiation assay. NB4 cells were labelled with isotype negative control (left) and CD11b (right) antibody. The non-specific antibody binding was excluded using the isotype negative antibody (left histogram-D-). According to the level of CD11b expression in the right histogram, the fluorescent intensity of 10^1 was determined as the cut off for differentiation. To quantify the cell differentiation, the percentage of $CD11b^{high}$ cells (signals higher than 10^1) was analysed.



		Control	48 hours	72 hours	96 hours	120 hours
Bright CD11b (%)	**ATRA**	2.66	48.08	57.98	75.49	83.49
	ATO	2.66	4.29	6.96	11.98	20.62

Figure 6.4. Cell differentiation assay. NB4 cells were treated with ATRA (0.5 µM) or ATO (0.5 µM), and CD11b expression was analysed by flow cytometry at various time points for up to 120 hours. Each peak with different colour represents the expression of CD11b at different time points compared to the control (red peak). The table below demonstrates the percentage of differentiated NB4 cells ($CD11b^{high}$) induced by ATRA and ATO.

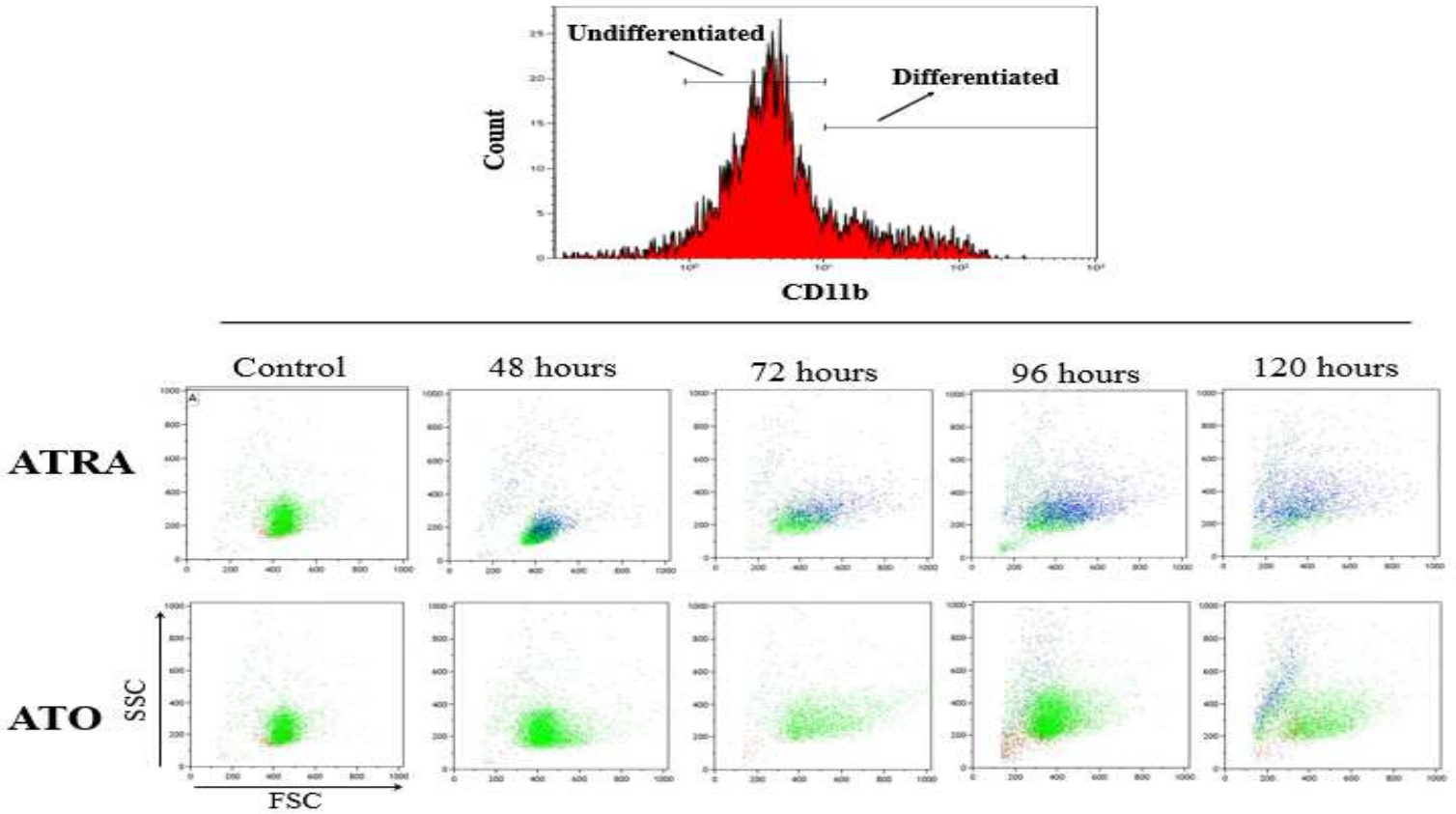


Figure 6.5. Representative cell differentiation assay at various time points. NB4 cells were treated with ATRA (0.5 µM) or ATO (0.5 µM), and CD11b expression was analysed by flow cytometry at various time points for up to 120 hours. Low and high CD11b expressing cells were determined using fluorescence intensity (Above demonstrative histogram). Changes in CD11b expression in different treatment conditions are demonstrated in below dot plots with different colours. **Green population** indicates non/low differentiated population ($CD11b^{neg/low}$), and **Blue population** indicates differentiated cells ($CD11b^{high}$).

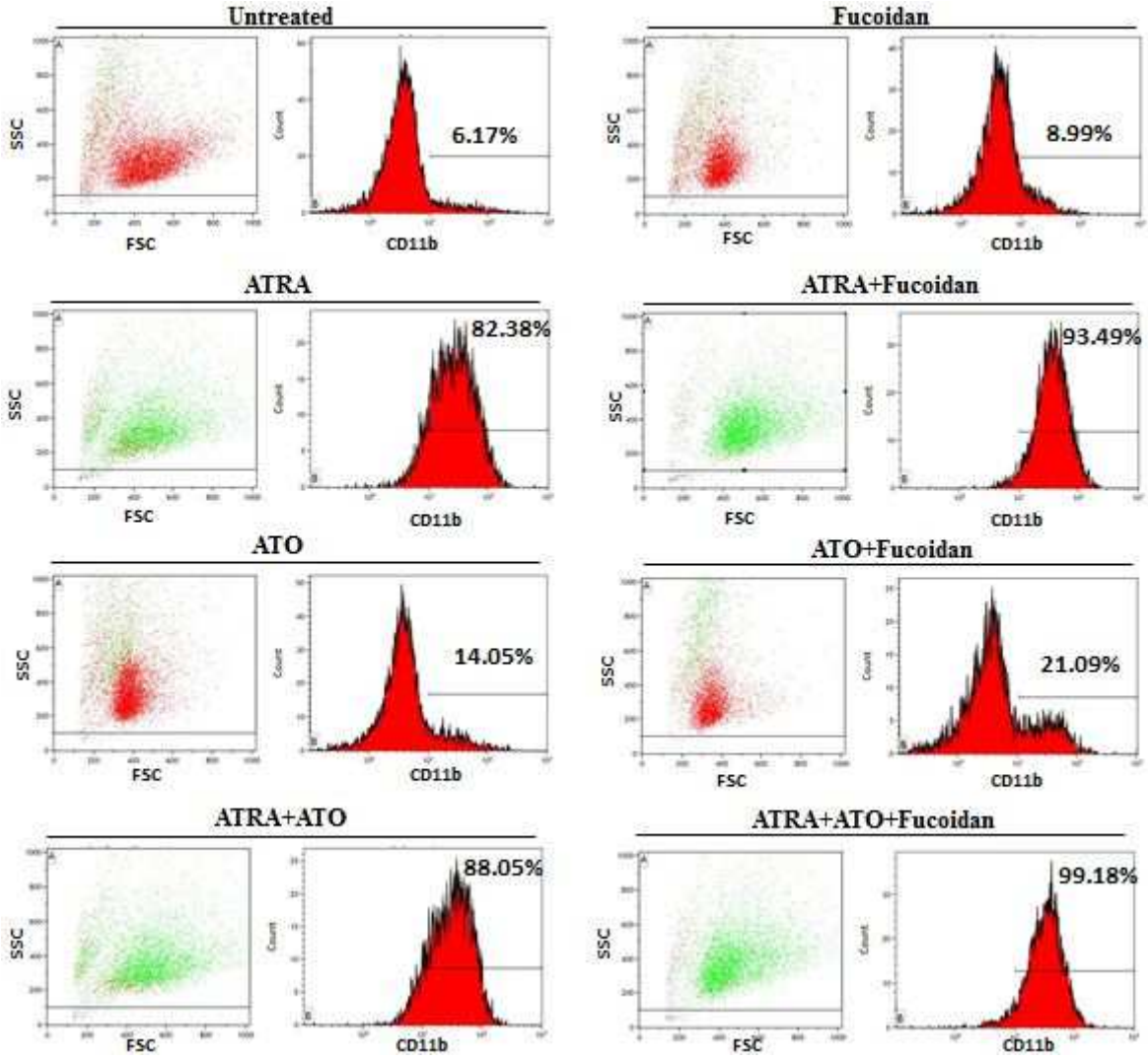


Figure 6.6. Representative cell differentiation analysis. NB4 cells were treated with ATRA alone (0.5 µM), ATO alone (0.5 µM), fucoidan alone(5 µg/mL) or in combination. CD11b expression was analysed by flow cytometry after 120 hours. $CD11b^{neg/low}$ cells were excluded, and the $CD11b^{high}$ population was calculated. In dot plot histograms, green population indicates differentiated population while red population represents non-differentiated cells.

Dot plot (left) histogram: Red population: non/low differentiated $CD11b^{neg/low}$ population, **Green population**: $CD11b^{hight}$ differentiated cells.

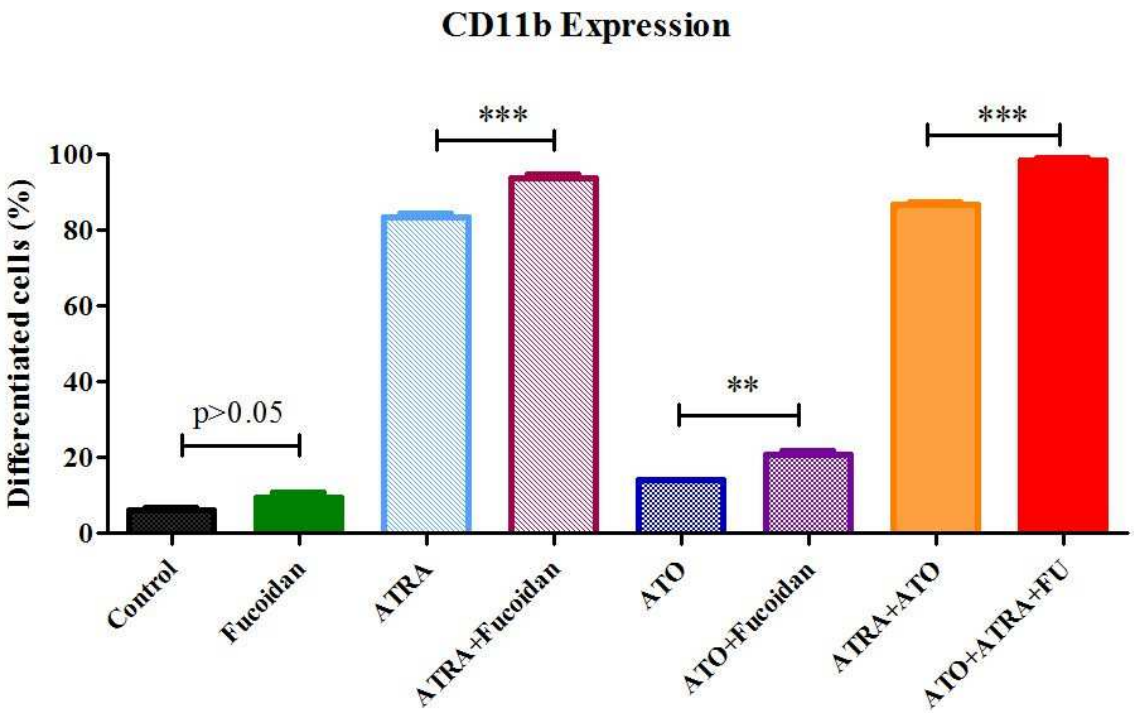


Figure 6.7. Effects of combination of fucoidan with ATRA and ATO on cell differentiation. NB4 cells were treated with ATRA alone (0.5 µM), ATO alone (0.5 µM), fucoidan alone (5 µg/mL) or in combination, and CD11b expression was analysed after 120 hours. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test (***: $p \leq 0.001$, **: $p \leq 0.01$).

6.3.2 Fucoidan Combined with ATRA and ATO Enhances G0/G1 arrest in APL Cells.

Cells undergoing differentiation normally stop dividing and exit the cell cycle dividing phases. Hence, the DNA content was assessed and the percentage of the non-dividing G0/G1 population, indicative of differentiation, was analysed in APL cells treated with single or combination of fucoidan, ATRA and ATO (Figure 6.8). Consistent with the observed changes in CD11b expression, a significant increase in the non-dividing G0/G1 population was observed in cells co-treated with ATRA+fucoidan compared to ATRA alone, and in cells co-treated with ATRA+ATO+fucoidan compared to ATRA+ATO (Table 6.1). Figure 6.9 compares the G0/G1 arrest in NB4 cells treated with different agents.

It should be noted that at the very low doses of fucoidan used, the amount of the sub G0/G1 dead cell population did not change in treated cells compared to the untreated cells (Table 6.1).

Table 6.1. Cell cycle phase distribution. NB4 cells were treated with ATRA (0.5 µM) and ATO (0.5 µM) with or without fucoidan (5 µg/mL), and DNA content was assessed using flow cytometry after 120 hours. Data represents mean ± SEM of at least three replicates.

		G0/G1	S	Mitosis
Agents alone	Control	60.64 ± 0.41	20.65 ± 1.1	18.07 ± 0.89
	Fucoidan	65.10 ± 1.54	20.77 ± 1.44	13.3 ± 0.10
	ATRA	76.01 ± 1.05	8.53 ± 1.00	15.12 ± 0.08
	ATO	64.40 ± 0.95	18.29 ± 1.29	16.64 ± 0.31
Combination	ATRA+fucoidan	84.28 ± 1.81	5.24 ± 0.21	10.24 ± 1.61
	ATO+fucoidan	66.01 ± 2.95	21.94 ± 1.77	11.38 ± 1.02
	ATRA + ATO	82.44 ± 0.53	6.78 ± 0.06	10.58 ± 0.65
	ATRA+ATO+fucoidan	89.86 ± 1.11	4.42 ± 0.94	5.41 ± 0.16

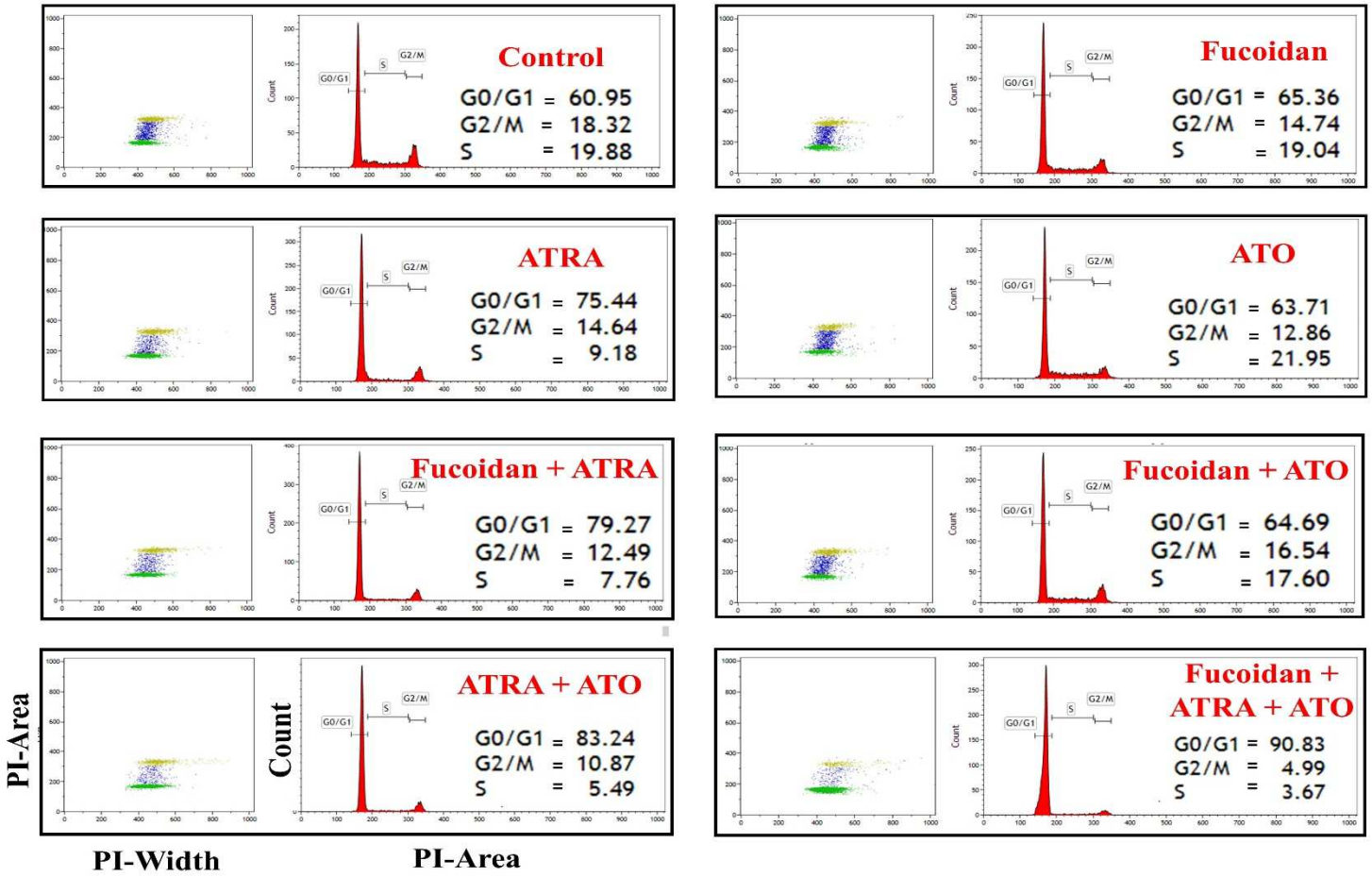


Figure 6.8. Representative cell cycle analysis. NB4 cells were treated with fucoidan alone (5 µg/mL), ATRA alone (0.5 µM), ATO alone (0.5 µM), or combinations of these agents. Cell cycle was assessed using flow cytometry. Results represent one of the three replicates. **Dot plot (left) histogram: Green population**: G0/G1 phase. **Blue population:** S phase. **Yellow population:** G2/M phase

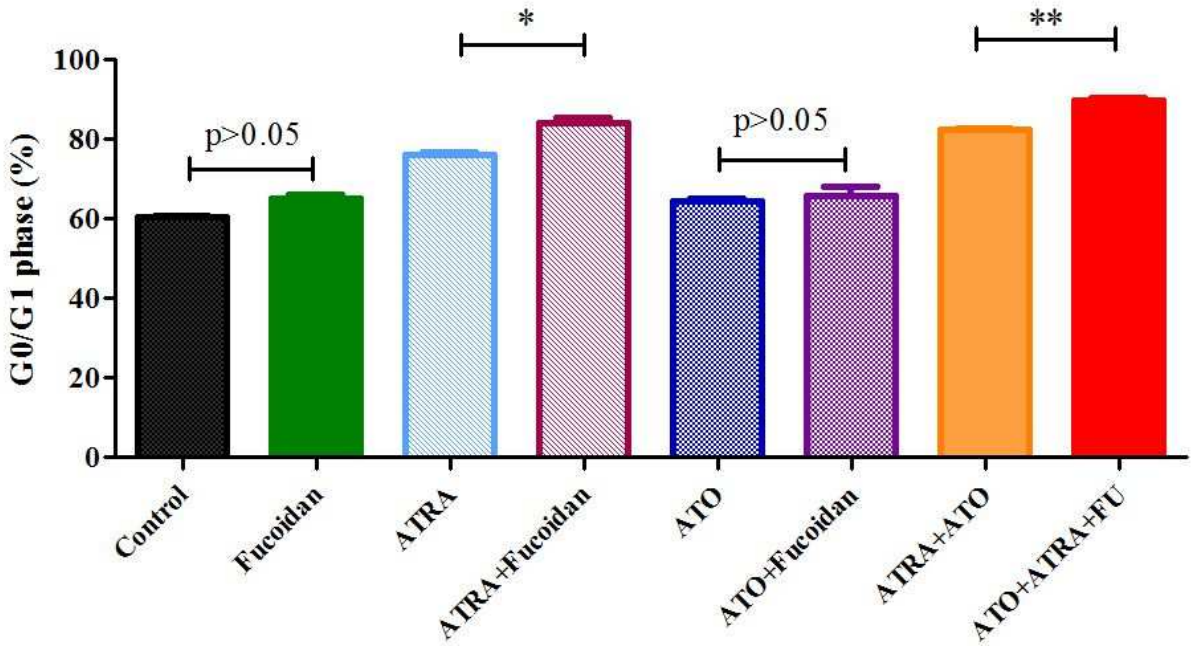


Figure 6.9. Effects of combination of fucoidan with ATRA and ATO on accumulation of the non-dividing G0/G1 cells. NB4 cells were treated with ATRA alone (0.5 µM), ATO alone (0.5 µM), fucoidan alone (5 µg/mL) or combinations of these agents, and DNA content was analysed after 120 hours. Mean ± SEM of at least three replicates is shown. Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test. (**: $p \leq 0.01$, *: $p \leq 0.05$)

6.3.3 Co-treatment of Fucoidan with ATRA Significantly Delayed the Growth of APL in Mice.

To evaluate the possible synergy of fucoidan and ATRA *in vivo*, a subcutaneous xenograft APL mass was established in 32 nude mice (n=8/group), and single or combined treatments of fucoidan and ATRA were commenced following tumour appearance. Tumour mass volume was monitored and median survival was identified using the Kaplan-Meier survival analysis.

A visible tumour mass (4~10 mm^3) appeared after 8-14 days in all 32 mice. During the treatment, none of the mice showed any side effects (e.g. weight loss or behavioural changes) to the administered doses. One animal in the fucoidan alone group and one animal in the ATRA alone group completed the full 28-days treatment. All remaining mice were sacrificed as per animal ethics guidelines prior to 28-days treatment as tumour volume reached 1000 mm^3.

Treatment with all three treated groups significantly slowed the increase in tumour mass with the tumours in these animals reaching the maximal end point much later than the control group. The median survival of the mice was 13, 19, 19 and 19.5 days in control, fucoidan alone, ATRA alone and combination groups, respectively (Figure 6.10). No significant difference was seen between treatment groups with each other.

Tumour aggressiveness was evaluated by tumour volume doubling time which decreased in all treatment groups compared to the control group. The mean tumour volume doubling time was 2.43, 3.92, 3.42 and 3.53 days in control, fucoidan, ATO and combination groups, respectively (Figure 6.11) indicating that the tumour in the control group grew more quickly than in treated mice. No significant difference was seen between treatment groups with each other.

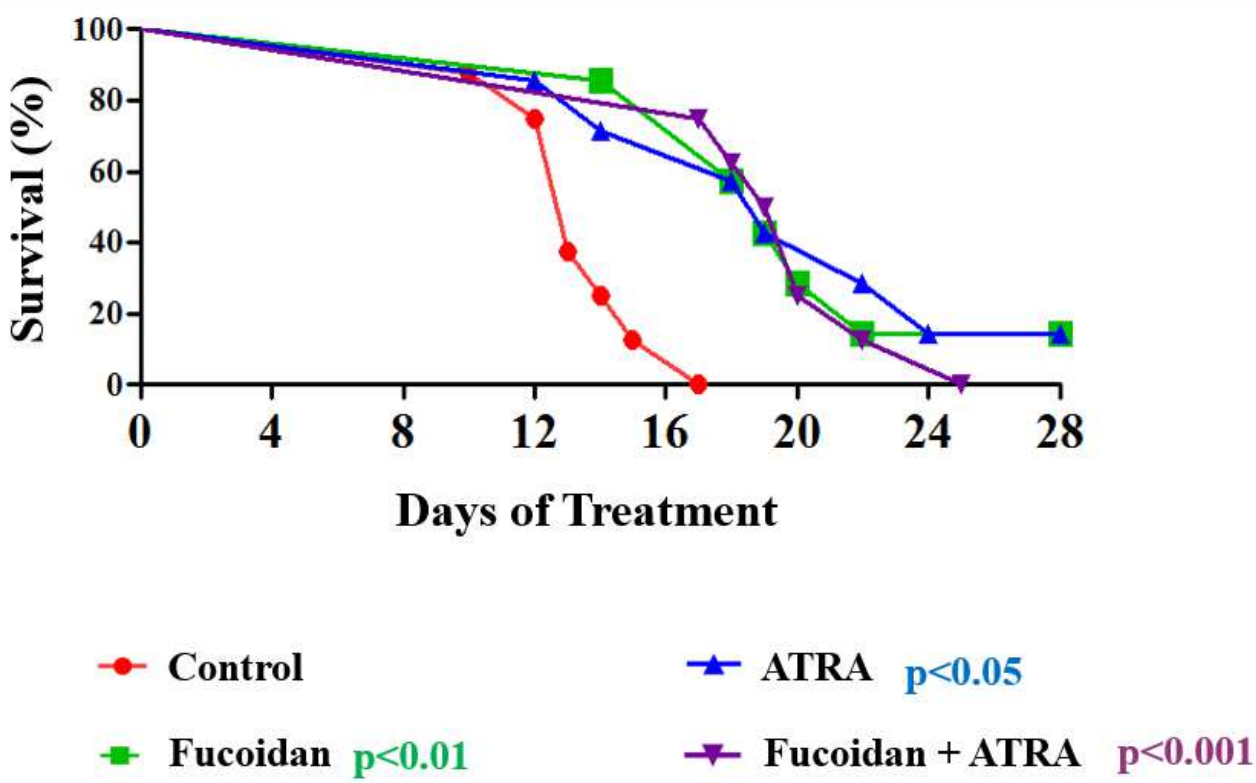


Figure 6.10. The Kaplan-Meier survival analysis over 28 days of treatment. Mice with APL were treated with vehicle (red), fucoidan (green), ATRA (blue) and their combination (purple) and their life span was analysed over 28 days of treatment (n=8/group). *p* values refer to comparison between each treatment group with the control group.

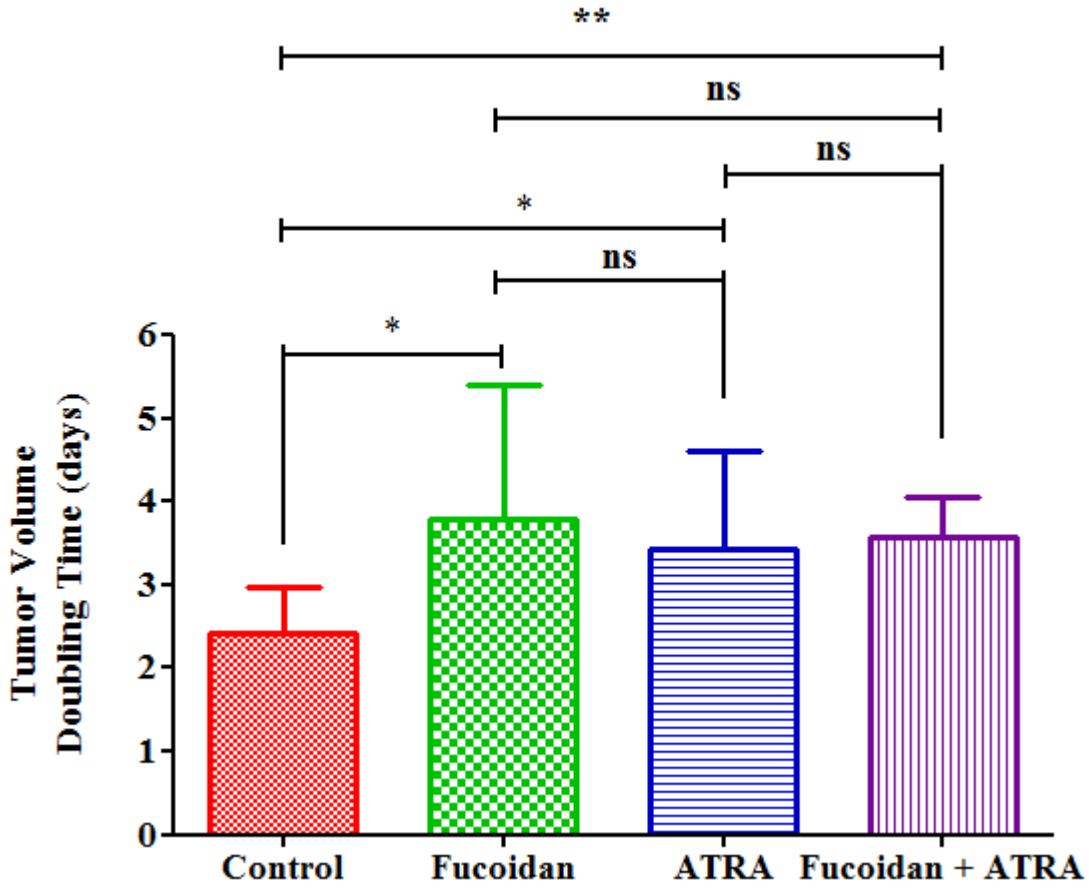


Figure 6.11. Mean tumour volume doubling time. Mice with APL were treated with vehicle (red), fucoidan (green), ATRA (blue) and their combination (purple) and their mean tumour doubling time was compared to that of the control group (n=8/group). Each column shows mean ± SD. Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test (**:$p \leq 0.01$, *: $p \leq 0.05$).

6.3.4 Fucoidan Significantly Increased the Differentiation of APL Cells *in Vivo*.

At the end of the *in-vivo* study of the combinatory effect of fucoidan plus ATRA, the tumour mass was removed from the mice and myeloid differentiation was assessed. To do this, a cell suspension from the removed tumour mass was prepared following labelling with anti-CD11b antibody. Since almost all NB4 cells express CD44, tumour cells were identified by CD44 expression. CD44 is an adhesion molecule which is highly expressed in more than 95% of the NB4 cells and its expression does not change upon differentiation and maturation. To analyse myeloid differentiation, $CD11b^{high}CD44^{+}$ cells were quantified.

Figure 6.12 shows representative CD11b expression histograms in tumour mass obtained from one representative mouse in each treatment group. As shown, a significant increase in differentiation was observed in tumour mass treated with fucoidan alone ($p \leq 0.05$) and ATRA+fucoidan ($p \leq 0.05$). The mean values of differentiated cells were approximately 10%, 24%, 19% and 22% in control, fucoidan, ATRA and combination groups, respectively (Figure 6.13). There was no significant difference between treated groups with each other.

Furthermore, a different immunophenotypic pattern in expression of CD44 and CD11b was observed in different treatment groups (Blue population in Figure 6.12). Evaluating the expression of CD44 on differentiated cells, the ratio of $CD11b^{high}CD44^{-}$ cells to $CD11b^{high}CD44^{+}$ cells was calculated (Figure 6.14). As shown in Figure 6.14, compared to the control group, the ratio was reversed in mice treated with ATRA+fucoidan, indicating that the percentage of CD44-negative differentiated cells was higher than the CD44-positive differentiated cells. This ratio was 0.58, 0.83, 0.87 and 1.14 in control, fucoidan alone, ATRA alone and combination groups, respectively, indicating decreased expression of CD44 in mice treated with fucoidan+ATRA.

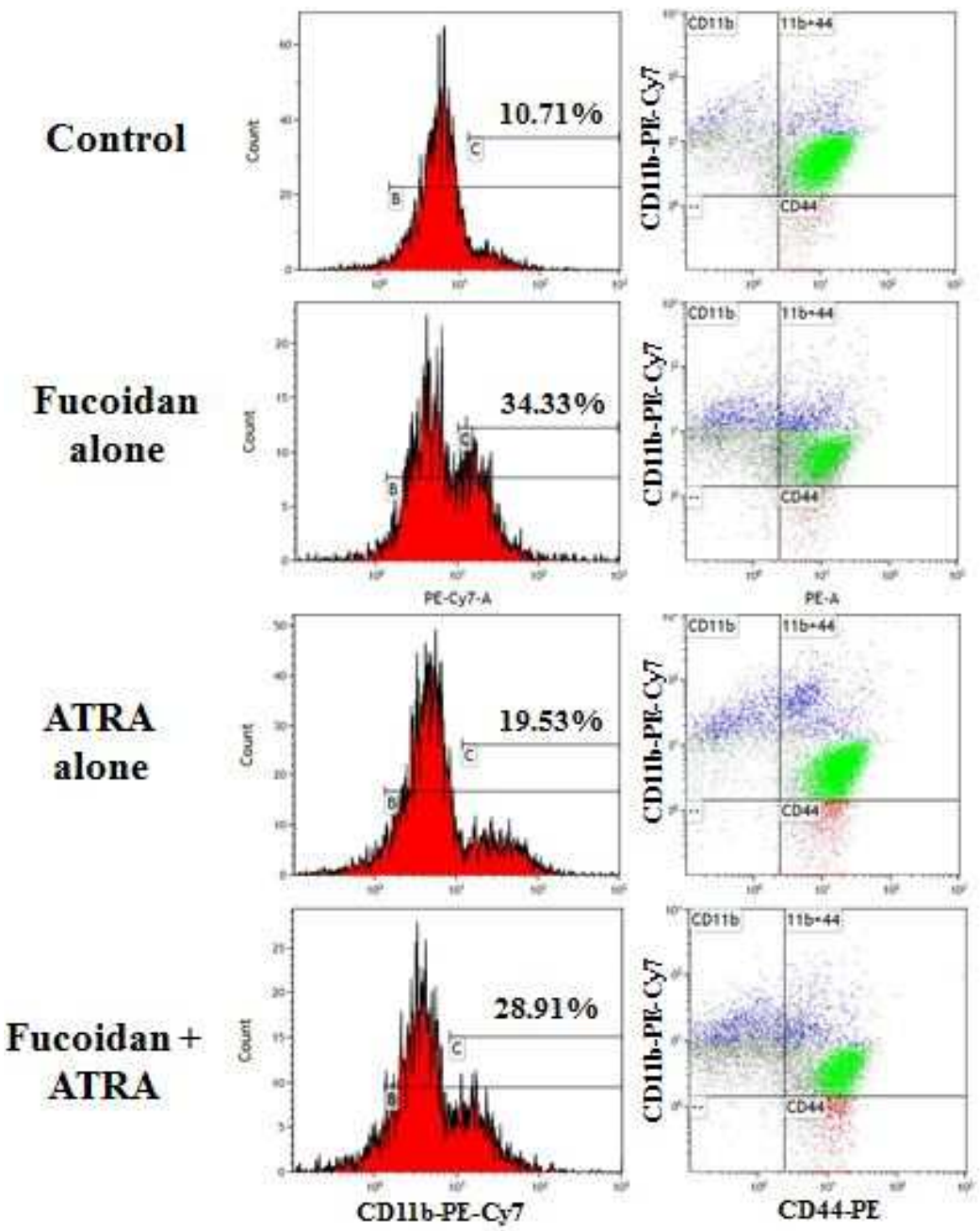


Figure 6.12. Representative flow cytometry analysis of CD11b expression in cells obtained from tumour mass. The tumour-bearing mice were treated with fucoidan, ATRA and combination, and differentiation was assessed using CD11b expression. Histograms show the flow cytometry results from one representative tumour mass in each treated group. The percentage of differentiated $CD11b^{high}$ cells was calculated (left graphs). The graphs in the right hand column demonstrate the expression of CD11b and CD44.
Blue population: $CD11b^{high}$, **Green and Gray populations**: $CD11b^{low}$
Red populations: $CD11b^{neg}$

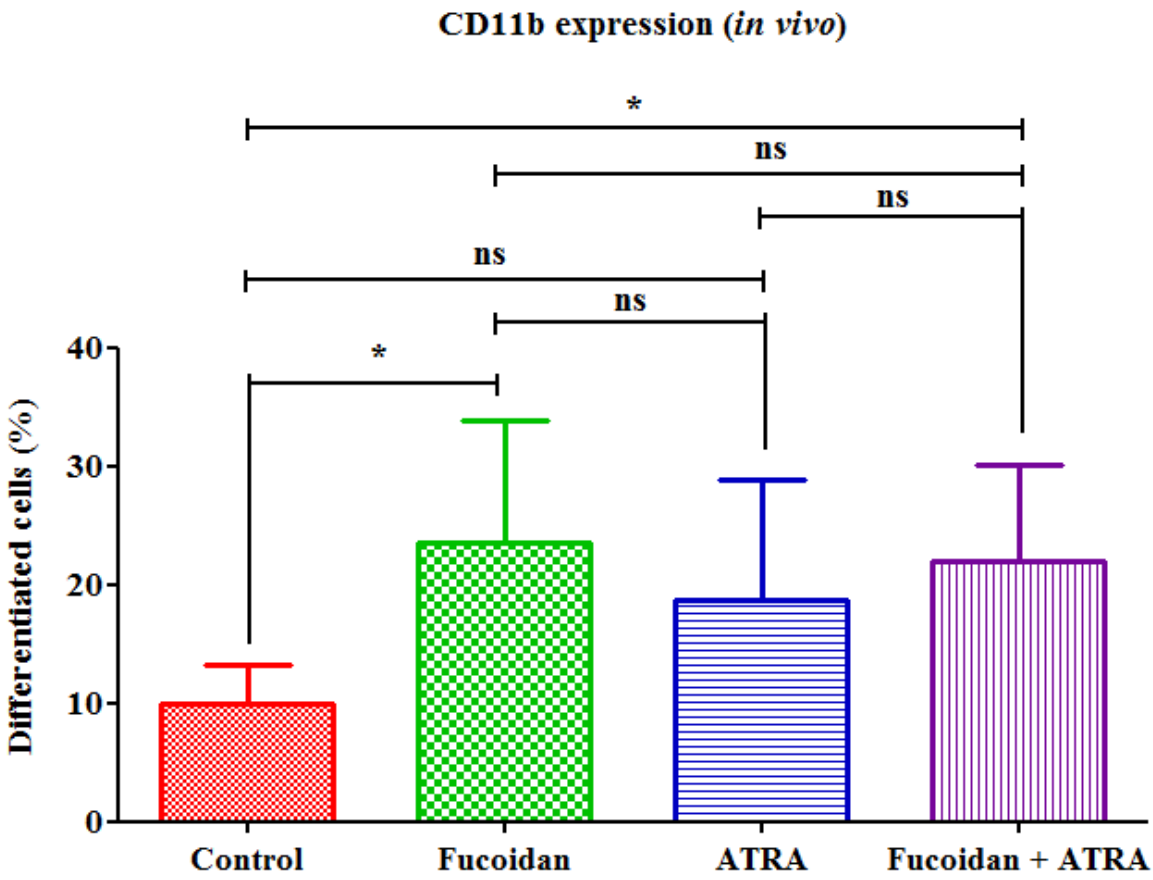


Figure 6.13. Myeloid differentiation in tumour mass obtained from mice treated with fucoidan, ATRA and combination compared to the control group. Differentiation was measured calculating $CD11b^{high}$ cells. Each column represents the mean ± SD of $CD11b^{high}$ cells in each experimental group (n=8/group). Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test (*: $p \leq 0.05$).

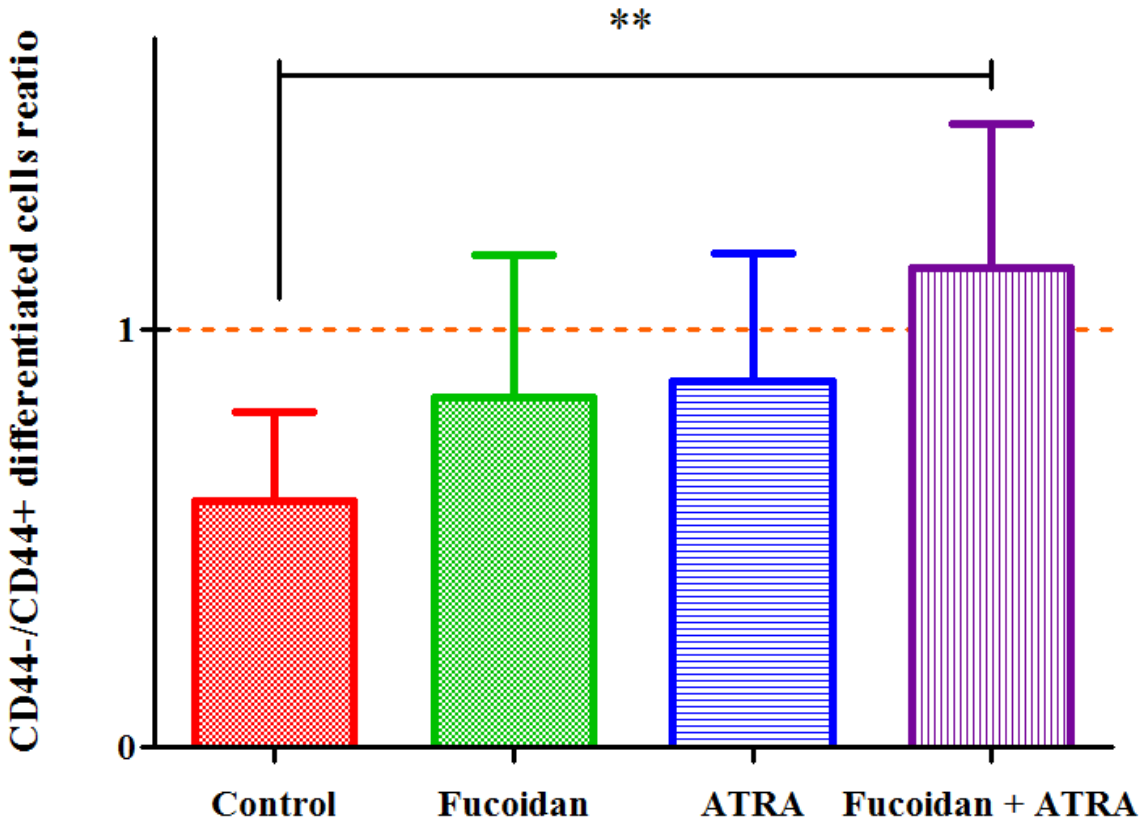


Figure 6.14. The ratio between $CD44^-/CD11b^+$ to $CD44^+/CD11b^+$ cells in tumour mass obtained from mice. Mice with APL were treated with vehicle, fucoidan, ATRA and combination. Expression of CD44 and CD11b was measured using flow cytometry and the ratio between CD44-negative to CD44-positive differentiated cells was calculated in each experimental group. Statistical significance was determined by ANOVA, followed by Tukey's multiple comparison test (**: $p \leq 0.01$). Each column represents the mean ratio of $CD44^-$ to $CD44^+$ differentiated cells with error bars the SD.

6.4 Discussion

Herein, the synergistic effects of fucoidan with ATRA and ATO in induction of myeloid differentiation was investigated. It was found that combining fucoidan with sub-therapeutic doses of ATRA and ATO enhanced myeloid differentiation *in vitro*. In addition, enhanced survival and delayed tumour growth were observed in APL-bearing mice when treated with combined fucoidan and low doses of ATRA. Analysis of myeloid differentiation in the tumour mass obtained from mice showed that treatment with fucoidan alone and combined with ATRA significantly increased tumour cell differentiation, *in vivo*. This is the first study to show that treatment of mice with fucoidan+ATRA down-regulates the expression of the adhesion molecule CD44 in APL cells.

6.4.1 Synergistic Effect of Fucoidan with ATRA and ATO *in Vitro*

Severe toxicity is frequently observed in newly diagnosed APL patients treated with traditional chemotherapeutic agents. Several studies and trials have investigated whether the combination of ATRA+ATO with minimal or no chemotherapy can replace standard therapy (308). Haematological remission is observed in 80-90% of refractory and relapsed patients treated with ATRA+ATO (272), however the use of this combination in newly diagnosed and high-risk patients remains clinically challenging and can induce serious complications such as hyperleucocytosis and differentiation syndrome (309, 310). Therefore, the use of adjuvants increasing the efficiency of ATO+ATRA on the one hand and decreasing the risk of side effects on the other gained strong interest.

Fucoidan's biological activities such as anti-tumour and immunomodulatory effects are highly relevant to its potential as an adjuvant therapy. In a recent study by Zuo *et al*., the role of fucoidan in minimising intestinal mucositis induced by chemotherapy was examined in mice (270). Mucositis is a common problem in patients under chemotherapy and is related to significant morbidity and rarely mortality. Zuo *et al.* developed a mouse model of mucositis induced by the chemotherapy drug cyclophosphamide and showed that administration of fucoidan significantly reduced the intestinal damage caused by chemotherapy. This was accompanied by an improved intestinal immune system as fucoidan altered the expression of intestinal immune system cytokines and maintained the Th1/Th2 immune balance. Fucoidan

has also been shown to increase the efficacy of chemotherapeutic agents. Zhang *et al*. reported that combining fucoidan with standard chemotherapeutic agents increased apoptosis in breast cancer cell lines, potentially through induction of oxidative stress (196). To the best of our knowledge, there is no study examining the combinatory effect of fucoidan with standard treatments in leukaemias.

Failure to differentiate is a feature of the abnormal promyelocytes in APL (286). ATRA and ATO are two main standard differentiation-based therapies which stimulate terminal and partial differentiation of the accumulated abnormal promyelocytes, respectively (40, 311). The selective growth inhibitory effect of fucoidan in APL cells was demonstrated in Chapter 3. Results in Chapter 5 also provided evidence that fucoidan has synergistic effect with standard APL therapy regimen (ATO) in induction of apoptosis. Here, further investigations were carried out to determine whether fucoidan can enhance the effectiveness of ATRA+ATO in induction of differentiation in APL cells.

CD11b or integrin alpha M (ITGAM) is the alpha subunit of the heterodimeric integrin alpha M beta 2 (αMβ2) protein which is also known as macrophage antigen-1 (Mac-1) or complement receptor 3 (CR3). This adhesion molecule is highly expressed on the surface of many leucocytes (312). The surface expression of CD11b is gradually increased upon the myeloid differentiation and maturation and hence its overexpression is an indicator of differentiation.

Previous studies have analysed the ATRA-mediated granulocytic differentiation of NB4 cells at various time points. Yang *et al*. analysed the expression profiles of more than 12,000 genes in NB4 cells after treatment with 1 μM ATRA at various time points (from 12 to 96 hours), and compared them to those of untreated cells (314). Differentiation markers began to increase after 24 hours with the maximum differentiation (more than 85%) at 96 hours after ATRA treatment. Here, the expression of CD11b was analysed in NB4 cells after treatment with ATRA from 48 to 120 hours, and the maximum differentiation was observed after 120 hours of treatment. The same analysis were conducted in NB4 cells treated with ATO. The granulocytic differentiation began 72-96 hours after the ATO treatment compared to the control. This is consistent with the literature as it is well documented that ATO induces only partial differentiation and slight changes in CD11b expression in NB4 cells compared to the terminal differentiation induced by ATRA (315).

Cell cycle assay is another tool for analysing cell differentiation. *In vitro*, proliferating cells are characterised by an active cell cycle leading to cellular divisions. With initiation of the differentiation process, cells exit from the dividing phases of the cell cycle. This is observed with decrease in the fraction of cells in S phase and mitosis phase parallel with increase in the non-dividing G0/G1 phase fraction (316). In this Chapter, the cell cycle status was measured to assess the differentiation after 120 hours. The amount of the S-phase population decreased from approximately 21% to 8% in the ATRA-treated NB4 cells. This decrease was consistent with the literature (314).

After the optimization assays, NB4 cells were treated with fucoidan plus sub-clinical doses of ATRA and ATO. In this chapter, an ultra-low dose of fucoidan was selected for the differentiation assay to minimise the fucoidan-mediated apoptosis in NB4 cells over the 120-hour incubation time. Co-stimulation of NB4 cells with fucoidan and ATRA+ATO resulted in synergistic induction of myeloid differentiation characterised by increased expression of CD11b and G0/G1 arrest. When fucoidan was combined with ATRA+ATO, almost all cells underwent differentiation. In contrast, when cells were treated with ATRA alone or ATRA+ATO, a proportion of cells remained undifferentiated. During chemotherapy, the drug-resistant cell population is the main source of cancer recurrence in patients. The findings of this thesis suggest that the number of resistant cells in response to ATRA could be limited in patients by addition of fucoidan to the standard APL regimen.

ATRA and ATO induce differentiation through the degradation of the oncoprotein PML-RARα, the main barrier of differentiation in APL cells (317). Previous studies on fucoidan's anti-cancer effects have shown that fucoidan mostly regulates the protein levels rather than mRNA levels. Hence it will be worthwhile to investigate the effects of fucoidan on the expression of PML-RARα fusion protein as it might alter the expression of the fusion protein or enhance its degradation through regulating the ubiquitin/proteasome pathway.

Although fucoidan significantly increased APL cell differentiation when it was combined with ATRA and ATO, fucoidan alone had almost no effect on NB4 cell differentiation *in vitro*. Reports examining differentiation response in other cell types have been reported, for example in osteoblasts and mesenchymal stem cells. In a recent study, fucoidan significantly increased differentiation in human mesenchymal stem cells at doses between 0.1-1 µg/mL after 120 hours. Differentiation was however markedly decreased at 5 and 10 µg/mL fucoidan (318).

Here, differentiation was not altered when cells were treated with lower or higher doses of fucoidan alone (1 µg/mL and 20 µg/mL) (data were not shown).

6.4.2 Synergistic Effect of Fucoidan with ATRA *in Vivo*

Following the *in vitro* findings, the synergistic effects of fucoidan with ATRA were examined *in vivo*. Several *in vivo* studies have reported side effects caused by high doses of ATO and ATRA. In a study by Jing *et al*., 10 µg/g ATRA increased the lifespan of mice bearing APL. However, combination of this dose of ATRA with ATO was toxic with weight loss and early therapy-associated death (40). Here, lower concentration of ATRA (1.5 µg/g b.w.) was employed, with the presumption that this may minimise the toxicities of the anti-cancer drugs. Due to small size of the utilised nude mice and risk of toxicity, administration of higher concentrations of ATRA was impossible. Knowing that previously published papers used at least 5 µg/gr b.w. of ATRA, at the beginning of the study a dose of 2.5 µg/gr b.w. of ATRA was selected for the *in vivo* investigations, but initiation of treatment led to the early death of animals where two mice died two days after treatment commencement (data were not shown). As a result the ATRA dose was further reduced to 1.5 µg/gr b.w. and observed no toxicity when it was used alone or in combination with fucoidan.

To address the effects of fucoidan on APL cell differentiation *in vivo*, the myeloid differentiation of the tumour mass obtained from mice treated with fucoidan and ATRA was lastly measured. In contrast with the *in vitro* findings, in which fucoidan alone had little effects on NB4 cell differentiation, *in vivo*, fucoidan alone induced 1.3-fold more differentiation in NB4 cells than did ATRA alone. Granulocytic differentiation includes a highly regulated process which occurs upon stimulation of several cytokines (319). The microenvironment and cellular cross talk are other critical factors that can influence the maturation and differentiation process. As fucoidan has been shown to change the production of various cytokines *in vivo* (320), it is suggested that the different results between fucoidan's action *in vivo* and *in vitro* could be explained by the effect of fucoidan on alteration of the cytokines that have regulatory effects on myeloid maturation. Fucoidan also affects the mobilization of the white blood cells from bone marrow to the peripheral blood through various mechanisms. Irhimeh *et al.* showed that fucoidan increased the expression of CXCR4 on haematopoietic stem cells resulting in the migration of these cells into the circulatory blood (321). Furthermore, it has been reported that

fucoidan has a synergistic effect with granulocyte colony stimulating factor (G-CSF) to alter leucocyte trafficking and mobilization (321). Changes in the pattern of circulatory blood cells combined with the altered stimulation of cytokine production by immune cells could lead to a different microenvironment around engrafted NB4 cells compared to *in-vitro*-cultured NB4 cells. G-CSF is a major cytokine, which increases granulocytic differentiation (322). Synergy of fucoidan with G-CSF in induction of differentiation is another hypothesis which needs to be investigated in future studies.

The combined fucoidan and ATRA treatment of mice bearing APL not only caused myeloid differentiation, but reduced CD44 expression. This was not observed in those mice treated with ATRA or fucoidan alone. Evaluating the expression of CD44 on differentiated cells, the ratio of $CD44^-$ cells to $CD44^+$ cells was measured, and it was found that when mice were treated with combined agents, the pattern of CD44 expression reversed upon maturation compared to the other groups. The observed reversal following combined ATRA+fucoidan treatment could be suggestive of two hypotheses: 1. the differentiating cells have lost their CD44 upon maturation 2. the existing $CD44^-$ cells have undergone higher amount of maturation than the existing $CD44^+$ cells. However, since more than 95% of NB4 cells express CD44 at high level, the second hypothesis seems implausible.

CD44 is an adhesion molecule involved in cell proliferation, differentiation, migration and angiogenesis (323). It is well documented that upregulation of CD44 on cancer cells is correlated with poor prognosis as it can enhance cell migration and metastasis (324). As mentioned above, development of the differentiation syndrome caused by ATRA and ATO is one of the main clinical challenges in treatment of APL, whereby the accumulated differentiated young neutrophils infiltrate into various body sites, particularly lungs, and cause fatal complications (325). The observed increase in differentiated cells with low or no expression of CD44 in mice treated with fucoidan+ATRA adds further evidence that it may decrease the migration of these cells. However, further analysis including developing murine models of differentiation syndrome following treatment with ATRA plus fucoidan is needed.

CHAPTER SEVEN

Conclusion and Future Directions

Final Discussion and Conclusion

This thesis focussed on examining the anti-tumour activity of the natural product, fucoidan, in haematological malignancies (HMs) both *in vitro* and *in vivo*. The chemical composition of fucoidan from different seaweed sources and their cytotoxicity activity on HMs were analysed and compared. *In-vitro* studies indicated that fucoidan selectively inhibited the growth of acute promyelocytic leukaemia (APL) cell lines through activation of apoptotic molecules and inactivation of signal transduction molecules involved in cell survival. The anti-tumour activity of fucoidan was supported by an *in-vivo* evidence which demonstrated that oral doses of fucoidan prior to inoculation with a human APL cell line significantly delayed tumour growth in the xenograft model, potentially by increasing the cytolytic activity of NK cells. In addition, significant synergy between fucoidan and anti-leukaemic activity of APL standard treatments ATO and ATRA was observed in the APL cells *in vitro*. *In vivo*, treatment of APL-bearing mice with fucoidan plus ATRA and fucoidan plus ATO resulted in delayed tumour growth and significantly prolonged the tumour volume doubling time compared to the control group.

There is considerable scope to improve treatment efficiency outcomes as the high mortality and morbidity associated with relapse, drug resistance and the risk of the development of therapy related cancers have remained unchanged in last decades. The challenges in the successful treatment of HMs arise largely because of the nature of the cancer. The dissemination of leukaemic cells in HMs limits the use of localised therapeutic strategies such as surgery and radiation (326). Further, by generating key growth and survival signals, bone marrow stroma provides a nurturing environment for leukaemic cells, leading to tumour progression, and resistance against the cytotoxic effects of chemotherapeutic agents (327).

Identification of novel and potent agents that target the cancer cell death while having minimum impact on normal cells is the main key toward developing successful therapeutic strategies. Thereby, agents with anti-cancer activity and reduced toxicity such as some natural products have attracted strong interest. The multifunctional properties of many natural products many of which are also known to be well tolerated make them ideal targets in anti-cancer research. Vinblastine and vincristine, for instance, are amongst the main chemotherapeutic agents, that are derived from the Madagascar periwinkle known as rosy periwinkle (328). The usage of these drugs in childhood leukaemia has improved the survival rate from 10% to 95%.

Eribulin is another natural-based anticancer agent, that has been developed from a sea sponge and its usage in metastatic breast cancer was approved by FDA in 2010 (329).

Fucoidan is a water soluble polysaccharide derived from brown seaweeds, and is consumed as a regular part of diet in Asian countries. Recent epidemiological studies have linked the consumption of seaweeds containing fucoidan with low risk of certain cancer types particularly female breast cancer (252). The biological properties of fucoidans derived from *Undaria pinnatifida* and *Fucus vesiculosus* have been under recent investigations. However, there are few studies on the growth inhibitory effects of these fucoidans in HMs. This is the first study to investigate the anti-tumour activity of fucoidan in HMs *in vivo*.

Different factors such as the source of fucoidan, the time and location of harvesting and the extraction method can affect the fucoidan's bioactivities (137). Fucoidan from two sources of *Undaria pinnatifida* and *Fucus vesiculosus* was used in this study. The purified fucoidan from *Undaria pinnatifida* and *Fucus vesiculosus* (Marinova Pty. Ltd) used in Chapter 3 are extracted by a neutral cold process and ultrafiltration. This process leaves the product with its native structure intact with a high molecular weight. These fucoidans were found to have no cytotoxic activity in any of the leukaemic cells utilised. Wang and colleagues extracted fucoidan from the sporophyll of *Undaria pinnatifida* using hot water extraction and alcohol grade precipitation (330). The product had a purity of >90% with the molecular weight of 104 kDa and contained 21% sulphate. At the dose of 250 µg/mL, this purified fucoidan inhibited growth in mouse hepatocarcinoma cells. The fucoidan from *Undaria pinnatifida* used in this thesis had almost similar characteristics to that of Wang *et al*., with a purity of 99%, MW of 61.2 kDa and a sulphate content of 26.6%. However, even at very high doses of 2.5 mg/mL it did not affect the leukaemia cell growth and cell cycle. This difference can possibly be explained by factors such as cell types and other structural characteristics.

Fucoidan from *Fucus vesiculosus* obtained from Sigma Co. was further utilised and tested. This *Fucus* fucoidan is the one most commonly reported to be used in research in this field. It is produced using acid hydrolysis and ethanol precipitation. The chemical constituent analysis in this thesis revealed structural differences between two fucoidan sources. For instance, the fucoidan from Sigma Co. had three times lower MW than that produced by Marinova Pty. Ltd. In addition, the purity of this fucoidan was near 80% compared to 98% in fucoidan from Marinova Pty. Ltd. In contrast with the purified fucoidan (Marinova Pty. Ltd.), the fucoidan

from Sigma Co. revealed strong cytotoxic activity in some AML cell lines. To ensure to avoid false results, the endotoxin level of the commercial fucoidan was measured and no endotoxin activity was detected.

The reason for the observed difference in results obtained from different fucoidans is not clear, although this has been noticed by others. Mak *et al.* isolated crude fucoidan from *Undaria pinnatifida* with 70% fucoidan purity and prepared three different fractions with higher purities, and compared the biological properties of these fractions with fucoidan from *Fucus vesiculosus* (Sigma Co.) (249). Cell proliferation assay in various cell lines showed that their own crude fucoidan had the highest cytotoxic activity than purified fractions and commercial fucoidan against the MCF-7 breast cancer cells and A-549 lung cancer cells. Another study by Choosawad *et al.* also reported a stronger inhibitory effect of crude fucoidan from *Utricularia aurea* on KB nasopharynx cancer cells than its purified fractions (331). It is generally believed that fucoidans with lower MW and higher sulphate content have higher growth inhibitory effect on cancer cells. However, in the Mak *et al.* report, the crude fucoidan had the highest MW and lowest sulphate content compared to those of the purified fractions but had the highest anti-cancer activity. Here, the different fucoidan preparations utilised had similar sulphate content. This could suggest that sulphate content in the range of 20% might not have a large impact on the fucoidan bio-properties.

Another difference between the three fucoidans studied in this thesis is the presence of 3.9% polyphenols in the fucoidan from Sigma Co. Polyphenols are phytochemicals widely distributed in plants and marine plants such as seaweeds and have been reported to have anti-oxidant and cytotoxic effects (237). It is not clear how much the presence of 3.9% polyphenols contributed to the cytotoxic effects of the fucoidan. In a recent study, Yang *et al*. showed that the polyphenol extracted from the brown seaweed *Laminaria japonica* Aresch had anti-proliferative activity on P388 leukaemia cell line with the IC_{50} value of >200 µg/mL ($p>0.05$) (332). To the best of our knowledge, the previously published articles studying the anti-cancer activity of fucoidan have not examined polyphenol content of their fucoidans.

Fucoidan has been shown to be non-toxic in normal tissues. For *in-vitro* studies, some researchers have utilised normal cells such as normal fibroblasts alongside tumour cell lines, and have examined the proliferation inhibitory effect of fucoidan on both tumour and normal cells. These studies have revealed that fucoidan did not induce apoptosis within normal cells

at the doses which were toxic for the studied cancer cell lines. *In vivo* and clinical trials using fucoidan from different sources have also reported no or mild side effects. For instance, long-term administration of fucoidan from *Undaria pinnatifida* in a form of 560 mg capsules did not induce side effects even when the participants took 4 capsules a day (333).

In this dissertation, it was determined that fucoidan from *Fucus vesiculosus* (Sigma Co.) effectively and selectively induced cell death in the APL cell lines NB4 and HL60 but did not have any inhibitory effect on proliferation of the minimally differentiated AML cell line KG-1a. It also slightly reduced the proliferation of the acute erythroleukaemic cell line K562 through induction of cell cycle arrest and not cell death. The induced cell death in APL cells was revealed to have features characteristic of apoptosis and not necrosis. Necrosis is a pathological cell death that causes inflammation upon the release of the cellular content in the surrounding environment. In contrast, during apoptosis the intracellular contents are not released and inflammation does not occur in the cellular environment. Therefore, the type of cell death induced by fucoidan makes it a desirable therapeutic agent.

Speculation of fucoidan's targets leading to its wide range of activities including cell death process in cancer cells is still a matter of debate and controversy. Here, fucoidan induced apoptosis through reducing the activation of survival molecules ERK1/2 and AKT in APL cells. It also hyper-activated the apoptosis enzymes caspases 8, 9 and 3, and its cytotoxic activity was attenuated using a pan-caspase inhibitor indicating that fucoidan's apoptosis induction is caspase dependent in APL cells.

Consumption of fucoidan even over a very short time period was found to significantly delay the development and growth of APL, suggesting that fucoidan intake may enhance the immune cell activity. Other studies have also shown that fucoidan can inhibit neoplastic transformation in other *in vivo* models. In a chemical carcinogen rat model, fucoidan delayed the median time for tumour appearance when fucoidan was intraperitoneally administrated for 34 days prior to carcinogen exposure (253). In a recent study, the prophylactic activity of oral fucoidan in the prevention of Lewis Lung Carcinoma cell invasion and metastasis was investigated (266). Fucoidan prophylactically inhibited lung metastasis colonisation of tumour cells in C57BL/6 mice.

Treatment with fucoidan alone has been shown to be effective in multiple cancer types *in vivo*. Many bioactive substances function to modulate multiple pathways including apoptosis, cell proliferation and differentiation. Therefore, use of combinations of such natural compounds with two or more targeted anti-cancer agents may achieve a more successful outcome.

APL is one of the most aggressive types of AML with the unusually young average age of 40 years. Despite the improvement in APL survival rate, drug resistance and side effects such as hyperleucocytosis and differentiation syndrome remain the clinical challenges. Here, the anti-tumour activity of fucoidan in different types of AML was examined, and it was found that fucoidan treatment inhibited APL cell proliferation. This finding led us to examine whether fucoidan combined with standard APL treatments would have therapeutic benefit.

A strong synergistic effect on apoptosis was noted in fucoidan and ATO treated cells. Combination treatment led to significant increase in DNA fragmentation and annexin V positive apoptotic cells. ATO plus ATRA traditionally targets the differentiation of malignant promyelocytes. As ATO can additionally induce apoptosis in APL cells, the involvement of ATO plus other therapeutic strategies in induction of apoptosis in promyelocytes is a target of cancer research studies. Treatment of human cervix carcinoma cells with ATO and ionised radiation enhanced apoptosis with loss of mitochondrial membrane potential and activation of caspases (334). The combination of ATO with the tyrosine kinase inhibitor imatinib also significantly increased chronic myelocytic leukaemic cell apoptosis through activation of the apoptosis intrinsic pathway, compared to stress-mediated apoptosis induced by ATO alone (335). ATO is currently administrated in refractory APL but not in newly diagnosed cases. This work is in agreement with other aforementioned studies (334, 335) that show more effective apoptotic action can be achieved using ATO plus other therapies rather than ATO alone. This observed synergy raises the possibility that ATO combined with other therapeutic agents such as fucoidan in newly diagnosed APL patients may be a viable option particularly arising from our observation of synergism between fucoidan with low doses of ATRA and ATO. These findings could be beneficial in clinics as the administration of lower doses of ATRA and ATO could reduce their observed toxicities.

The synergistic effect of fucoidan on APL cell differentiation induced by ATRA and ATO was further determined in the human APL cell line, NB4. Triple combinations of these agents were found to induce the strongest influence on cell cycle arrest compared to single or any double

combinations of the compounds. The cell cycle arrest observed was consistent with the data obtained from the differentiation assay and the triple combination resulted in the highest amount of differentiated cells where almost all cells underwent differentiation. In NB4 cells treated with dual combination of ATRA+ATO, a small proportion of resistant cells (~15%) remained undifferentiated. The primary cause of relapse is drug resistance and therefore incomplete clearance of tumour cells. The findings presented in this thesis have implications for improving treatment response in APL cells as the addition of fucoidan to standard ATRA+ATO therapy could overcome resistance and relapse in APL.

Although combination of low dose fucoidan with ATRA or ATO synergistically enhanced the differentiation, no dual dose dependent effect was observed for fucoidan alone treatment such as was seen in ATO-treated APL cells. As mentioned before, ATO induces apoptosis at higher doses while it induces myeloid differentiation at lower doses. In contrast, fucoidan only induced cell death at higher dose but did not induce differentiation at the lower dose when it was administered alone.

Finally, the synergy of fucoidan+ATO or fucoidan+ATRA was investigated *in vivo*. The combination of ATRA plus fucoidan retarded tumour growth as indicated by increased tumour volume doubling time. However, overall the maximal effect was observed in fucoidan+ATO treated mice. Approximately 28% of the mice treated with fucoidan+ATO completed the full treatment program, with tumour volume reaching the end point after 28 days compared to none in fucoidan+ATRA treated mice. Furthermore, the tumour volume doubling time was maximal in fucoidan+ATO treated mice compared to all other groups in all studies. This could be because of the very low dose of ATRA used here due to toxicity encountered as described previously. This low dose may explain the lower efficacy of fucoidan+ATRA compared to fucoidan+ATO treatment and to the *in vitro* results.

The anti-tumour activity of fucoidan treatment as a single agent was demonstrated, and consistent outcomes were achieved. Further, in combination with ATRA and ATO, fucoidan was again demonstrated to potentiate the anti-tumour activities. The results obtained from the optimisation assay (see Section 5.3.3), the fucoidan+ATO assay (see Section 5.3.4) and the fucoidan+ATRA assay (see Section 6.3.3) reveal the anti-tumour activity of fucoidan alone treatment compared to the control group in APL-bearing mice. Figure 7.1 compares the tumour volume doubling time and the median survival in fucoidan alone treated mice and the control

group in two independent assays in Chapters 5 and 6. As shown, almost similar outcomes were observed in the fucoidan treated mice in both studies. The reproducibility and observed consistent anti-cancer activity of fucoidan when used alone strengthens the hypothesis that fucoidan has anti-tumour activity against APL.

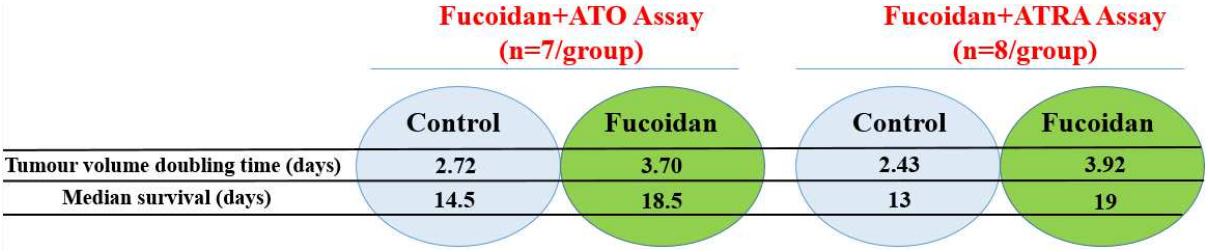


Figure 7.1. Comparison of the data obtained in two independent studies in Chapters 5 and 6. Results show tumour volume doubling time and median survival in the control (blue) and fucoidan alone (green) treated mice from studies in Chapter 5 (left) and Chapter 6 (right).

When the differentiation activity of fucoidan treatment alone was assessed, myeloid differentiation of APL cells was observed *in vivo*, while no such effect was observed *in vitro*. We believe that fucoidan treatment alters the microenvironment around engrafted NB4 cells compared to *in-vitro*-cultured NB4 cells. Fucoidan's influences on cell cross-talk and cytokine production *in vivo* may explain the induced differentiation in APL cells in mice.

Another novel observation was to identify the altered ratio of $CD44^-$ cells to $CD44^+$ cells in the tumour masses of those mice treated with fucoidan+ATRA compared to single treatment and control groups. It is not clear whether co-treatment of ATRA with fucoidan reduced the expression of CD44 upon differentiation or induced differentiation of existing $CD44^-$ NB4 cells more than the $CD44^+$ NB4 cells. Although as it is known that almost 95% of the NB4 cells express CD44 on their surface, reduction in CD44 expression appears plausible. CD44 plays a major role in migration of tumour cells to other sites of the body and therefore downregulation of CD44 by combined fucoidan and ATRA may reduce the migration of APL cells in differentiation syndrome caused by ATRA and ATO. Although, this remains to be proven and further investigation on the underpinning mechanisms of this action should be undertaken. This could be analysed by labelling the $CD44^-$ NB4 cells prior to injection into mice.

Future Directions

This thesis provides the first evidence that the combination of fucoidan with APL standard therapy delays APL growth and enhances the survival of APL-bearing mice. More animal experiments using larger numbers and additional treatment groups such as a triple combination of fucoidan, ATRA and ATO are suggested. Also, future studies which examine systemic leukaemia closely mimicking circulatory leukaemia cells in humans should be undertaken with establishment of APL via injection of the tumour cells intravenously. The use of NOD/SCID mice would permit tumour burden to be examined in the absence of NK cells, B-cells and T-cells, and tumour establishment would be more successful. NOD-SCID mice can also tolerate higher doses of ATRA compared to nude mice.

The research presented in this thesis also provides evidences for immunomodulatory activity of fucoidan through changes in NK cell behaviour *in vivo*. Whether the increased activity of NK cells were due to increased numbers or function remains to be determined. In addition, the role of fucoidan on other immune response elements such as IFN- level could be examined in future studies.

As it is hypothesised that fucoidan reduces the toxicity of ATRA and ATO through its immunomodulatory effects, it is worthwhile to analyse the protective activity of fucoidan on differentiation syndrome which is one of the main features of ATRA and ATO therapies. This could be done by establishment of differentiation syndrome model in mice bearing systemic APL treated with high dose ATRA, and assessing specific outcomes such as lung edema and pulmonary effusion which are indicators of differentiation syndrome.

This thesis also provides solid evidence of synergy of fucoidan plus ATO plus ATRA *in vitro* in NB4 cell differentiation. The combination of fucoidan+ATRA+ATO resulted in myeloid differentiation of >99% of treated cells compared to 87% when cells were treated with ATRA+ATO. ATRA and ATO induce differentiation through degradation of the oncoprotein PML-RARα, the main barrier of differentiation in APL cells (317). Further future investigations to examine the effects of fucoidan on the expression of PML-RARα fusion protein are suggested.

This work provides evidence about the protective effect of fucoidan in prevention and delayed growth of APL. These data have implications for future epidemiological studies. Randomised

controlled trials may seek to evaluate the effect of dietary fucoidan supplementation or brown seaweed intake on leukaemia prevention. These data could also be a basis for clinical trials in order to assess the association between oral administration of fucoidan and immune system functions such as NK cell activity as was shown in this project.

References

1. American Cancer Society. Cancer Facts & Figures 2014. Atlanta: American Cancer Society 2014.

2. Australian Institute of Health and Welfare 2014. Cancer in Australia, An overview 2014. Cancer Series no 90 Cat no CAN 88 Canberra: AIHW.

3. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114(5):937-51.

4. Juliusson G, Lazarevic V, Horstedt AS, Hagberg O, Hoglund M. Acute myeloid leukemia in the real world: why population-based registries are needed. Blood. 2012;119(17):3890-9.

5. Australian Institute of Health and Welfare & Australasian Association of Cancer Registries 2012. Cancer in Australia: An overview 2012. Cancer series no 74 Cat no CAN 70 Canberra: AIHW.

6. Hutchison RE, Schexneider KI. Leukocytic disorders. In: McPherson RA, Pincus MR, editors. Henry's Clinical Diagnosis and Management by Laboratory Methods. 22 ed2011.

7. Australian Institute of Health and Welfare 2012. Cancer survival and prevalence in Australia: period estimates from 1982 to 2010. Cancer Series no 69 Cat no CAN 65 Canberra: AIHW.

8. Deschler B, Lubbert M. Acute myeloid leukemia: epidemiology and etiology. Cancer. 2006;107(9):2099-107.

9. Schiffer CA, Stone RM. Acute Myeloid Leukemia in Adults. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast RC, Gansler TS, Holland JF, et al., editors. Holland-Frei Cancer Medicine. 6 ed: Hamilton (ON): BC Decker; 2003. p. 1.

10. Samiev D, Bhatt VR, Armitage JD, Maness LJ, Akhtari M. A primary care approach to myelodysplastic syndromes. Korean journal of family medicine. 2014;35(3):111-8.

11. Padua RA, McGlynn A, McGlynn H. Molecular, cytogenetic and genetic abnormalities in MDS and secondary AML. Cancer treatment and research. 2001;108:111-57.

12. Häggström M. Hematopoiesis. In: Image:Hematopoiesis_(human)_diagram.png, editor. Wikipedia2009.

13. SEER (Surveillance Epidemiology and End Results). Cancer Statistics Review, 1975-2011. National Cancer Institute; 2014.

14. Schlenk RF. Post-remission therapy for acute myeloid leukemia. Haematologica. 2014;99(11):1663-70.

15. Ravandi F, Estey EH, Appelbaum FR, Lo-Coco F, Schiffer CA, Larson RA, et al. Gemtuzumab ozogamicin: time to resurrect? Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2012;30(32):3921-3.

16. Avvisati G, Mele A, Stazi MA, Vegna ML, Pasquini P, Mandelli F. Epidemiology of acute promyelocytic leukemia in Italy. APL Collaborating Group. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 1991;2(6):405-8.

17. Hillestad LK. Acute promyelocytic leukemia. Acta medica Scandinavica. 1957;159(3):189-94.

18. Bernard J, Mathe G, Boulay J, Ceoard B, Chome J. [Acute promyelocytic leukemia: a study made on 20 cases]. Schweizerische medizinische Wochenschrift. 1959;89:604-8.

19. Roussel MJ, Lanotte M. Maturation sensitive and resistant t(15;17) NB4 cell lines as tools for APL physiopathology: nomenclature of cells and repertory of their known genetic alterations and phenotypes. Oncogene. 2001;20(49):7287-91.

20. Rowley JD, Golomb HM, Dougherty C. 15/17 translocation, a consistent chromosomal change in acute promyelocytic leukaemia. Lancet. 1977;1(8010):549-50.

21. Pandolfi PP. Oncogenes and tumor suppressors in the molecular pathogenesis of acute promyelocytic leukemia. Human molecular genetics. 2001;10(7):769-75.

22. Tallman MS, Altman JK. Curative strategies in acute promyelocytic leukemia. Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program. 2008;2008:391-9.

23. Palmer M, Chan A, Dieckmann T, Honek T. Cell type-specific anticancer drugs. In: Palmer M, Chan A, Dieckmann T, Honek T, editors. Notes to biochemical pharmacology: Wiley; 2013.

24. Tallman MS, Nabhan C, Feusner JH, Rowe JM. Acute promyelocytic leukemia: evolving therapeutic strategies. Blood. 2002;99(3):759-67.

25. Sanz MA, Jarque I, Martin G, Lorenzo I, Martinez J, Rafecas J, et al. Acute promyelocytic leukemia. Therapy results and prognostic factors. Cancer. 1988;61(1):7-13.

26. Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood. 2008;111(5):2505-15.

27. Iland HJ, Collins M, Bradstock K, Supple SG, Catalano A, Hertzberg M, et al. Use of arsenic trioxide in remission induction and consolidation therapy for acute promyelocytic leukaemia in the Australasian Leukaemia and Lymphoma Group (ALLG) APML4 study: a non-randomised phase 2 trial. The Lancet Haematology. 2015;2(9):e357-66.

28. Özpolat B. Acute promyelocytic leukemia and differentiation therapy: molecular mechanisms of differentiation, retinoic acid resistance and novel treatments. Turkish Journal of Hematology. 2009;26:47-61.

29. Lotan R. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochimica et biophysica acta. 1980;605(1):33-91.

30. Huang ME, Ye YC, Chen SR, Zhao JC, Gu LJ, Cai JR, et al. All-trans retinoic acid with or without low dose cytosine arabinoside in acute promyelocytic leukemia. Report of 6 cases. Chin Med J (Engl). 1987;100(12):949-53.

31. Lo Coco F, Nervi C, Avvisati G, Mandelli F. Acute promyelocytic leukemia: a curable disease. Leukemia. 1998;12(12):1866-80.

32. Warrell RP, Jr. Retinoid resistance in acute promyelocytic leukemia: new mechanisms, strategies, and implications. Blood. 1993;82(7):1949-53.

33. Montesinos P, Sanz MA. The differentiation syndrome in patients with acute promyelocytic leukemia: experience of the pethema group and review of the literature. Mediterranean journal of hematology and infectious diseases. 2011;3(1):e2011059.

34. Liu J, Lu Y, Wu Q, Goyer RA, Waalkes MP. Mineral arsenicals in traditional medicines: orpiment, realgar, and arsenolite. The Journal of pharmacology and experimental therapeutics. 2008;326(2):363-8.

35. Zhu J, Chen Z, Lallemand-Breitenbach V, de The H. How acute promyelocytic leukaemia revived arsenic. Nature reviews Cancer. 2002;2(9):705-13.

36. Sun HD, Ma L, Hu XC, Zhang TD. Ai-lin 1 treated 32 cases of acute promyelocytic leukemia. Chinese Journal of Integrative Medicine. 1992;12:170-1.

37. Antman KH. Introduction: the history of arsenic trioxide in cancer therapy. The oncologist. 2001;6 Suppl 2:1-2.

38. Mayorga J, Richardson-Hardin C, Dicke KA. Arsenic trioxide as effective therapy for relapsed acute promyelocytic leukemia. Clinical journal of oncology nursing. 2002;6(6):341-6.

39. Falchi L, Verstovsek S, Ravandi-Kashani F, Kantarjian HM. The evolution of arsenic in the treatment of acute promyelocytic leukemia and other myeloid neoplasms: Moving toward an effective oral, outpatient therapy. Cancer. 2015.

40. Jing Y, Wang L, Xia L, Chen GQ, Chen Z, Miller WH, et al. Combined effect of all-trans retinoic acid and arsenic trioxide in acute promyelocytic leukemia cells in vitro and in vivo. Blood. 2001;97(1):264-9.

41. Lallemand-Breitenbach V, Guillemin MC, Janin A, Daniel MT, Degos L, Kogan SC, et al. Retinoic acid and arsenic synergize to eradicate leukemic cells in a mouse model of acute promyelocytic leukemia. The Journal of experimental medicine. 1999;189(7):1043-52.

42. Shen ZX, Shi ZZ, Fang J, Gu BW, Li JM, Zhu YM, et al. All-trans retinoic acid/As2O3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(15):5328-35.

43. Tallman MS. Treatment of relapsed or refractory acute promyelocytic leukemia. Best practice & research Clinical haematology. 2007;20(1):57-65.

44. Long ZJ, Hu Y, Li XD, He Y, Xiao RZ, Fang ZG, et al. ATO/ATRA/anthracycline-chemotherapy sequential consolidation achieves long-term efficacy in primary acute promyelocytic leukemia. PloS one. 2014;9(8):e104610.

45. Lo-Coco F, Avvisati G, Vignetti M, Thiede C, Orlando SM, Iacobelli S, et al. Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. The New England journal of medicine. 2013;369(2):111-21.

46. Zhu HH, Wu DP, Jin J, Li JY, Ma J, Wang JX, et al. Oral tetra-arsenic tetra-sulfide formula versus intravenous arsenic trioxide as first-line treatment of acute promyelocytic leukemia: a multicenter randomized controlled trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2013;31(33):4215-21.

47. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.

48. Pecorino L. Introduction; what is cancer. Molecular Biology of Cancer: Mechanisms, Targets, and Therapeutics: Oxford University Press; 2012. p. 3.

49. Nath S, Mukherjee P. MUC1: a multifaceted oncoprotein with a key role in cancer progression. Trends in molecular medicine. 2014;20(6):332-42.

50. Xing X, Lian S, Hu Y, Li Z, Zhang L, Wen X, et al. Phosphatase of regenerating liver-3 (PRL-3) is associated with metastasis and poor prognosis in gastric carcinoma. Journal of translational medicine. 2013;11:309.

51. Kerr J, Wyllie A, Currie A. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer. 1972;26(4):239-57.

52. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516.

53. Abadeer A. Cell Death and Cell Renewal. The Cell: A Molecular Approach. 5 ed: Sinauer Associates, Inc.; 2011.

54. Kam P, Ferch N. Apoptosis: mechanisms and clinical implications. Anaesthesia. 2000;55(11):1081-93.

55. Lawen A. Apoptosis-an introduction. BioEssays. 2003;25(9):888-96.

56. http://www.hixonparvo.info/model.html. Apoptosis Pathway. In: apoptosis.jpg, editor.

57. Favaloro B, Allocati N, Graziano V, Di Ilio C, De Laurenzi V. Role of apoptosis in disease. Aging. 2012;4(5):330-49.

58. Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, et al. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science (New York, NY). 1997;278(5336):294-8.

59. Gewies A. Introduction to Apoptosis 2003 [July 2015]. Available from: http://www.ihcworld.com/_books/apointro.pdf.

60. Kasibhatla S, Tseng B. Why target apoptosis in cancer treatment? Molecular cancer therapeutics. 2003;2(6):573-80.

61. Wilkinson J, Cepero E, Boise LH, Duckett C. Upstream regulatory role for XIAP in receptor-mediated apoptosis. Molecular and cellular biology. 2004;24(16):7003-14.

62. Johnson P, Watt S, Betts D, Davies D, Jordan S, Norton A, et al. Isolated follicular lymphoma cells are resistant to apoptosis and can be grown in vitro in the CD40/stromal cell system. Blood. 1993;82(6):1848-57.

63. Ott G, Rosenwald A. Molecular pathogenesis of follicular lymphoma. Haematologica. 2008;93(12):1773-6.

64. Inoue K, Kohno T, Takakura S, Hayashi Y, Mizoguchi H, Yokota J. Frequent microsatellite instability and BAX mutations in T cell acute lymphoblastic leukemia cell lines. Leukemia research. 2000;24(3):255-62.

65. Devarajan E, Sahin A, Chen JS, Krishnamurthy R, Aggarwal N, Brun A, et al. Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. Oncogene. 2002;21(57):8843-51.

66. Takeuchi S, Takeuchi N, Fermin A, Taguchi H, Koeffler H. Frameshift mutations in caspase-5 and other target genes in leukemia and lymphoma cell lines having microsatellite instability. Leukemia research. 2003;27(4):359-61.

67. Ozoren N, El-Deiry WS. Cell surface Death Receptor signaling in normal and cancer cells. Seminars in cancer biology. 2003;13(2):135-47.

68. Gronbaek K, Straten P, Ralfkiaer E, Ahrenkiel V, Andersen M, Hansen N, et al. Somatic Fas mutations in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity. Blood. 1998;92(9):3018-24.

69. Searle J, Lawson T, Abbott P, Harmon B, Kerr J. An electron-microscope study of the mode of cell death induced by cancer-chemotherapeutic agents in populations of proliferating normal and neoplastic cells. The Journal of pathology. 1975;116(3):129-38.

70. Greaney P, Nahimana A, Lagopoulos L, Etter A, Aubry D, Attinger A, et al. A Fas agonist induces high levels of apoptosis in haematological malignancies. Leukemia research. 2006;30(4):415-26.

71. Nahimana A, Aubry D, Lagopoulos L, Greaney P, Attinger A, Demotz S, et al. A novel potent Fas agonist for selective depletion of tumor cells in hematopoietic transplants. Blood cancer journal. 2011;1(12):e47.

72. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, et al. Lethal effect of the anti-Fas antibody in mice. Nature. 1993;364(6440):806-9.

73. Havell E, Fiers W, North R. The antitumor function of tumor necrosis factor (TNF), I. Therapeutic action of TNF against an established murine sarcoma is indirect, immunologically dependent, and limited by severe toxicity. The Journal of experimental medicine. 1988;167(3):1067-85.

74. Wang S, Ren W, Liu J, Lahat G, Torres K, Lopez G, et al. TRAIL and doxorubicin combination induces proapoptotic and antiangiogenic effects in soft tissue sarcoma in vivo. Clinical Cancer Research. 2010;16(9):2591-604.

75. Borthakur G, O'Brien S. Pharmacology and clinical potential of oblimersen sodium in the treatment of chronic lymphocytic leukemia. Blood and Lymphatic Cancer: Targets and Therapy. 2012;2:137-43.

76. Kline M, Rajkumar S, Timm M, Kimlinger T, Haug J, Lust J, et al. ABT-737, an inhibitor of Bcl-2 family proteins, is a potent inducer of apoptosis in multiple myeloma cells. Leukemia. 2007;21(7):1549-60.

77. Cang S, Iragavarapu C, Savooji J, Song Y, Liu D. ABT-199 (venetoclax) and BCL-2 inhibitors in clinical development. Journal of hematology & oncology. 2015;8(1):129.

78. Davids MS, Pagel JM, Kahl B, .S., Wierda WG, Miller TP, Gerecitano JF, et al. Bcl-2 Inhibitor ABT-199 (GDC-0199) Monotherapy Shows Anti-Tumor Activity Including Complete Remissions In High-Risk Relapsed/Refractory (R/R) Chronic Lymphocytic Leukemia (CLL) and Small Lymphocytic Lymphoma (SLL). Blood. Nov 2013;122(21):872.

79. Peterson Q, Goode D, West D, Ramsey K, Lee JJ, Hergenrother P. PAC-1 Activates Procaspase-3 in Vitro through Relief of Zinc-Mediated Inhibition. Journal of molecular biology. 2009;388(1):144-58.

80. Tamm I, Wagner M, Schmelz K. Decitabine activates specific caspases downstream of p73 in myeloid leukemia. Annals of hematology. 2005;84 Suppl 1:47-53.

81. Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Overview of the Cell Cycle and Its Control. Molecular Cell Biology. 4 ed. New York: W. H. Freeman; 2000.

82. Sbia M, Parnell EJ, Yu Y, Olsen AE, Kretschmann KL, Voth WP, et al. Regulation of the yeast Ace2 transcription factor during the cell cycle. The Journal of biological chemistry. 2008;283(17):11135-45.

83. Vats P, Yu J, Rothfield L. The dynamic nature of the bacterial cytoskeleton. Cellular and molecular life sciences : CMLS. 2009;66(20):3353-62.

84. Jayat C, Ratinaud MH. Cell cycle analysis by flow cytometry: principles and applications. Biol Cell. 1993;78(1-2):15-25.

85. Malumbres M, Barbacid M. Milestones in cell division : To cycle or not to cycle: a critical decision in cancer. Nature Reviews Cancer. 2001;1(December):222-31.

86. Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G. Cyclin A is required at two points in the human cell cycle. The EMBO Journal. 1992;11(3):961-71.

87. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. The Journal of biological chemistry. 1996;271(34):20608-16.

88. Gladkikh A, Potashnikova D, Korneva E, Khudoleeva O, Vorobjev I. Cyclin D1 expression in B-cell lymphomas. Experimental hematology. 2010;38(11):1047-57.

89. Scuderi R, Palucka KA, Pokrovskaja K, Bjorkholm M, Wiman KG, Pisa P. Cyclin E overexpression in relapsed adult acute lymphoblastic leukemias of B-cell lineage. Blood. 1996;87(8):3360-7.

90. Iida H, Towatari M, Tanimoto M, Morishita Y, Kodera Y, Saito H. Overexpression of cyclin E in acute myelogenous leukemia. Blood. 1997;90(9):3707-13.

91. Della Ragione F, Mercurio C, Iolascon A. Cell cycle regulation and human leukemias: the role of p16INK4 gene inactivation in the development of human acute lymphoblastic leukemia. Haematologica. 1995;80(6):557-68.

92. Ogawa S, Hirano N, Sato N, Takahashi T, Hangaishi A, Tanaka K, et al. Homozygous loss of the cyclin-dependent kinase 4-inhibitor (p16) gene in human leukemias. Blood. 1994;84(8):2431-5.

93. Byrd JC, Lin TS, Dalton JT, Wu D, Phelps MA, Fischer B, et al. Flavopiridol administered using a pharmacologically derived schedule is associated with marked clinical efficacy in refractory, genetically high-risk chronic lymphocytic leukemia. Blood. 2007;109(2):399-404.

94. Lacrima K, Valentini A, Lambertini C, Taborelli M, Rinaldi A, Zucca E, et al. In vitro activity of cyclin-dependent kinase inhibitor CYC202 (Seliciclib, R-roscovitine) in mantle cell lymphomas. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2005;16(7):1169-76.

95. Berg J, ymoczko JL, Stryer L. Signal-Transduction Pathways: An Introduction to Information Metabolism. Biochemistry. 5 ed. New York: W.H. Freeman; 2002.

96. Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. Oncogene. 2007;26(22):3279-90.

97. Giehl K, Skripczynski B, Mansard A, Menke A, Gierschik P. Growth factor-dependent activation of the Ras-Raf-MEK-MAPK pathway in the human pancreatic carcinoma cell line

PANC-1 carrying activated K-ras: implications for cell proliferation and cell migration. Oncogene. 2000;19(25):2930-42.

98. Hemmings BA, Restuccia DF. The PI3K-PKB/Akt Pathway. Cold Spring Harbor perspectives in biology. 2015;7(4).

99. Robey RB, Hay N. Is Akt the "Warburg kinase"?-Akt-energy metabolism interactions and oncogenesis. Seminars in cancer biology. 2009;19(1):25-31.

100. Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, et al. BRAF and RAS mutations in human lung cancer and melanoma. Cancer research. 2002;62(23):6997-7000.

101. Hallek M, Bergsagel PL, Anderson KC. Multiple myeloma: increasing evidence for a multistep transformation process. Blood. 1998;91(1):3-21.

102. Janssen JW, Steenvoorden AC, Lyons J, Anger B, Bohlke JU, Bos JL, et al. RAS gene mutations in acute and chronic myelocytic leukemias, chronic myeloproliferative disorders, and myelodysplastic syndromes. Proceedings of the National Academy of Sciences of the United States of America. 1987;84(24):9228-32.

103. Park S, Chapuis N, Tamburini J, Bardet V, Cornillet-Lefebvre P, Willems L, et al. Role of the PI3K/AKT and mTOR signaling pathways in acute myeloid leukemia. Haematologica. 2010;95(5):819-28.

104. Blackburn JS, Liu S, Wilder JL, Dobrinski KP, Lobbardi R, Moore FE, et al. Clonal evolution enhances leukemia-propagating cell frequency in T cell acute lymphoblastic leukemia through Akt/mTORC1 pathway activation. Cancer cell. 2014;25(3):366-78.

105. Cohen PR, Bedikian AY, Kim KB. Appearance of New Vemurafenib-associated Melanocytic Nevi on Normal-appearing Skin: Case Series and a Review of Changing or New Pigmented Lesions in Patients with Metastatic Malignant Melanoma After Initiating Treatment with Vemurafenib. The Journal of clinical and aesthetic dermatology. 2013;6(5):27-37.

106. Lancet JE, Gojo I, Gotlib J, Feldman EJ, Greer J, Liesveld JL, et al. A phase 2 study of the farnesyltransferase inhibitor tipifarnib in poor-risk and elderly patients with previously untreated acute myelogenous leukemia. Blood. 2007;109(4):1387-94.

107. Janku F, Tsimberidou AM, Garrido-Laguna I, Wang X, Luthra R, Hong DS, et al. PIK3CA mutations in patients with advanced cancers treated with PI3K/AKT/mTOR axis inhibitors. Molecular cancer therapeutics. 2011;10(3):558-65.

108. Khan KH, Yap TA, Yan L, Cunningham D. Targeting the PI3K-AKT-mTOR signaling network in cancer. Chinese journal of cancer. 2013;32(5):253-65.

109. Furman RR, Sharman JP, Coutre SE, Cheson BD, Pagel JM, Hillmen P, et al. Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. The New England journal of medicine. 2014;370(11):997-1007.

110. Forcello N, Saraiya N. Idelalisib: The First-in-Class Phosphatidylinositol 3-Kinase Inhibitor for Relapsed CLL, SLL, and Indolent NHL. Journal of the advanced practitioner in oncology. 2014;5(6):455-9.

111. Finn OJ. Immuno-oncology: understanding the function and dysfunction of the immune system in cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2012;23 Suppl 8:viii6-9.

112. Highfill SL, Cui Y, Giles AJ, Smith JP, Zhang H, Morse E, et al. Disruption of CXCR2-mediated MDSC tumor trafficking enhances anti-PD1 efficacy. Science translational medicine. 2014;6(237):237ra67.

113. Wang SY, Racila E, Taylor RP, Weiner GJ. NK-cell activation and antibody-dependent cellular cytotoxicity induced by rituximab-coated target cells is inhibited by the C3b component of complement. Blood. 2008;111(3):1456-63.

114. O'Day SJ, Hamid O, Urba WJ. Targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4): a novel strategy for the treatment of melanoma and other malignancies. Cancer. 2007;110(12):2614-27.

115. Wolchok JD, Saenger Y. The mechanism of anti-CTLA-4 activity and the negative regulation of T-cell activation. The oncologist. 2008;13 Suppl 4:2-9.

116. Philips GK, Atkins M. Therapeutic uses of anti-PD-1 and anti-PD-L1 antibodies. International immunology. 2015;27(1):39-46.

117. Terunuma H, Deng X, Dewan Z, Fujimoto S, Yamamoto N. Potential role of NK cells in the induction of immune responses: implications for NK cell-based immunotherapy for cancers and viral infections. International reviews of immunology. 2008;27(3):93-110.

118. Smyth MJ, Cretney E, Kershaw MH, Hayakawa Y. Cytokines in cancer immunity and immunotherapy. Immunological reviews. 2004;202:275-93.

119. Huang V, Hepper D, Anadkat M, Cornelius L. Cutaneous toxic effects associated with vemurafenib and inhibition of the BRAF pathway. Archives of dermatology. 2012;148(5):628-33.

120. Dotan E, Aggarwal C, Smith MR. Impact of Rituximab (Rituxan) on the Treatment of B-Cell Non-Hodgkin's Lymphoma. P & T : a peer-reviewed journal for formulary management. 2010;35(3):148-57.

121. Khazir J, Riley DL, Pilcher LA, De-Maayer P, Mir BA. Anticancer agents from diverse natural sources. Natural product communications. 2014;9(11):1655-69.

122. Kingston DG. The shape of things to come: structural and synthetic studies of taxol and related compounds. Phytochemistry. 2007;68(14):1844-54.

123. George SK, Rajesh R, Kumar SS, Sulekha B, Balaram P. A polyherbal ayurvedic drug--Indukantha Ghritha as an adjuvant to cancer chemotherapy via immunomodulation. Immunobiology. 2008;213(8):641-9.

124. Caliceti P, Salmaso S, Bersani S. Polysaccharide-Based Anticancer Prodrugs. In: Reddy LH, Couvreur P, editors. Macromolecular Anticancer Therapeutics: Springer New York; 2010. p. 163-6.

125. Aravind SR, Joseph MM, Varghese S, Balaram P, Sreelekha TT. Antitumor and immunopotentiating activity of polysaccharide PST001 isolated from the seed kernel of Tamarindus indica: an in vivo study in mice. TheScientificWorldJournal. 2012;2012:361382.

126. Atashrazm F, Lowenthal RM, Woods GM, Holloway AF, Dickinson JL. Fucoidan and Cancer: A Multifunctional Molecule with Anti-Tumor Potential. Marine drugs. 2015;13(4):2327-46.

127. Jimenez-Escrig A, Goni Cambrodon I. Nutritional evaluation and physiological effects of edible seaweeds. Archivos latinoamericanos de nutricion. 1999;49(2):114-20.

128. Irhimeh M.R. Clinical haemopoietic implications of fucoidan treatment. Australia: University of Tasmania; 2008.

129. Vasseur E. Chemical studies on the jelly coat of the sea-urchin egg. Acta Chemistry Scandinavia. 1948;2:900-13.

130. Mulloy B, Ribeiro A, Alves A, Vieira R, Mourao P. Sulfated fucans from echinoderms have a regular tetrasaccharide repeating unit defined by specific patterns of sulfation at the O-2 and O-4 positions. J Biol Chem. 1994(269):22113-23.

131. Ribeiro A, Vieira R, Mourao P, Mulloy B. A sulfated a-L-fucan from sea cucumber. Carbohydrate Research. 1994; 255:225-40.

132. Bo Li B, Lu F, Wei X, Zhao R. Fucoidan: Structure and Bioactivity. Molecules (Basel, Switzerland). 2008;13:16711695.

133. Mak WWF. Extraction, Characterization and Antioxidant Activity of Fucoidan from New Zealand Undaria pinnatifida (Harvey) Suringar: Auckland University of Technology; 2012.

134. Frenette P, Weiss L. Sulfated glycans induce rapid hematopoietic progenitor cell mobilization: evidence for selectin dependent and independent mechanisms. Blood. 2000;96:2460-8.

135. Lee JB, Hayashi K, Hashimoto M, Nakano T, Hayashi T. Novel antiviral fucoidan from sporophyll of Undaria pinnatifida (Mekabu). Chemical & pharmaceutical bulletin (Tokyo). 2004;52(9):1091-4.

136. Fitton JH. Therapies from fucoidan; multifunctional marine polymers. Marine drugs. 2011;9(10):1731-60.

137. Mabeau S, Kloareg B, Joseleau J. Fractionation and analysis of fucans from brown algae. Phytochemistry. 1990;29:2441-5.

138. Black WAP. The seasonal variation in the combined L-fucose content of the common British Laminariaceae and fucaceae. J Sci Food Agr. 1954;5(9):445-8.

139. Kloareg B, Demarty M, Mabeau S. Polyanionic characteristic of purified sulphated homofucans from brown algae. International journal of biological macromolecules. 1986;8:380-6.

140. Curiel D, Bellemo G, Marzocchi M, Scattolin M, Parisi G. Distribution of introduced Japanese macroalgae Undaria pinnatifida, Sargassum muticum (Phaeophyta) and Antithamnion pectinatum (Rhodophyta) in the Lagoon ofVenice. Hydrobiologia. 1998;385:17-22.

141. Castric-Fey A, Beaupoil C, Bouchain J, Pradier E, L'Hardy-Halos M. The introduced alga Undaria pinnatifida (Laminariales, Alariaceae) in the rocky shore ecosystem of the St. Malo area: growth rate and longevity of the sporophyte. Botanica Marina. 1999;42:83-96.

142. Fletcher R, Manfredi C. The occurrence of Undaria pinnatifida (Phaeophyceae, Laminariales) on the south coast of England. Botanica Marina. 1995;38:355–8.

143. Hay C, Luckens P. The Asian kelp Undaria pinnatifida (Phaeophyta: Laminariales) found in a New Zealand Harbour. New Zealand Journal of Botany. 1987;25:329-32.

144. Casas G, Piriz M. Surveys of Undaria pinnatifida (Laminariales, Phaeophyta) in Golfo Nuevo, Argentina. Hydrobiologia. 1996; 326/327:231–15.

145. Campbell S, Bite J, Burridge T. Seasonal patterns in the photosynthetic capacity, tissue pigment, and nutrient content of different developmental stages of Undaria pinnatifida (Phaeophyta: Laminariales) in Port Phillip Bay, South-Eastern Australia. Botanica Marina. 1999;42:231–41.

146. Sanderson J. A preliminary survey of the introduced macroalga, Undaria pinnatifida (Harvey) Suringar on the east coast of Tasmania. Botanica Marina. 1990;33:153–7.

147. Silchenko AS, Kusaykin MI, Kurilenko VV, Zakharenko AM, Isakov VV, Zaporozhets TS, et al. Hydrolysis of fucoidan by fucoidanase isolated from the marine bacterium, Formosa algae. Marine drugs. 2013;11(7):2413-30.

148. Irhimeh MR, Fitton JH, Lowenthal RM, Kongtawelert P. A quantitative method to detect fucoidan in human plasma using a novel antibody. Methods and findings in experimental and clinical pharmacology. 2005;27(10):705-10.

149. Tokita Y, Nakajima K, Mochida H, Iha M, Nagamine T. Development of a fucoidan-specific antibody and measurement of fucoidan in serum and urine by sandwich ELISA. Bioscience, biotechnology, and biochemistry. 2010;74(2):350-7.

150. Deux JF, Meddahi-Pelle A, Le Blanche AF, Feldman LJ, Colliec-Jouault S, Bree F, et al. Low molecular weight fucoidan prevents neointimal hyperplasia in rabbit iliac artery in-stent restenosis model. Arteriosclerosis, thrombosis, and vascular biology. 2002;22(10):1604-9.

151. Kimura R, Rokkaku T, Takeda S, Senba M, Mori N. Cytotoxic effects of fucoidan nanoparticles against osteosarcoma. Marine drugs. 2013;11(11):4267-78.

152. Thelen T, Hao Y, Medeiros AI, Curtis JL, Serezani CH, Kobzik L, et al. The class A scavenger receptor, macrophage receptor with collagenous structure, is the major phagocytic receptor for Clostridium sordellii expressed by human decidual macrophages. Journal of immunology (Baltimore, Md : 1950). 2010;185(7):4328-35.

153. Yamasaki Y, Yamasaki M, Tachibana H, Yamada K. Important role of beta1-integrin in fucoidan-induced apoptosis via caspase-8 activation. Bioscience, biotechnology, and biochemistry. 2012;76(6):1163-8.

154. Ding Z, Issekutz TB, Downey GP, Waddell TK. L-selectin stimulation enhances functional expression of surface CXCR4 in lymphocytes: implications for cellular activation during adhesion and migration. Blood. 2003;101(11):4245-52.

155. Aisa Y, Miyakawa Y, Nakazato T, Shibata H, Saito K, Ikeda Y, et al. Fucoidan induces apoptosis of human HS-sultan cells accompanied by activation of caspase-3 and down-regulation of ERK pathways. Am J Hematol. 2005;78(1):7-14.

156. Zhu XD, Zhuang Y, Ben JJ, Qian LL, Huang HP, Bai H, et al. Caveolae-dependent endocytosis is required for class A macrophage scavenger receptor-mediated apoptosis in macrophages. The Journal of biological chemistry. 2011;286(10):8231-9.

157. Kim M, Joo H. Immunostimulatory effects of fucoidan on bone marrow-derived dendritic cells. Immunology Letters. 2008;115(2):138-43.

158. Zhao X, Xue C, Cai Y, Wang D, Fang Y. The study of antioxidant activities of fucoidan from Laminaria japonica. High Technology Letters. 2005;11:91-4.

159. Kwak JY. Fucoidan as a marine anticancer agent in preclinical development. Marine drugs. 2014;12(2):851-70.

160. Zhang Z, Teruya K, Eto H, Shirahata S. Fucoidan extract induces apoptosis in MCF-7 cells via a mechanism involving the ROS-dependent JNK activation and mitochondria-mediated pathways. PloS one. 2011;6(11):e27441.

161. Riou D, Colliec-Jouault S, Pinczon du Sel D, Bosch S, Siavoshian S, Le Bert V, et al. Antitumor and antiproliferative effects of a fucan extracted from ascophyllum nodosum against a non-small-cell bronchopulmonary carcinoma line. Anticancer research. 1996;16(3a):1213-8.

162. Moreau D, Thomas-Guyon H, Jacquot C, Jugé M, Culioli G, Ortalo-Magné A, et al. An extract from the brown alga Bifurcaria bifurcata induces irreversible arrest of cell proliferation

in a non-small-cell bronchopulmonary carcinoma line. Journal of applied phycology. 2006;18:87-93.

163. Boo HJ, Hong JY, Kim SC, Kang JI, Kim MK, Kim EJ, et al. The anticancer effect of fucoidan in PC-3 prostate cancer cells. Marine drugs. 2013;11(8):2982-99.

164. Banafa AM, Roshan S, Liu YY, Chen HJ, Chen MJ, Yang GX, et al. Fucoidan induces G1 phase arrest and apoptosis through caspases-dependent pathway and ROS induction in human breast cancer MCF-7 cells. Journal of Huazhong University of Science and Technology Medical sciences = Hua zhong ke ji da xue xue bao Yi xue Ying De wen ban = Huazhong keji daxue xuebao Yixue Yingdewen ban. 2013;33(5):717-24.

165. Haneji K, Matsuda T, Tomita M, Kawakami H, Ohshiro K, Uchihara JN, et al. Fucoidan extracted from Cladosiphon okamuranus Tokida induces apoptosis of human T-cell leukemia virus type 1-infected T-cell lines and primary adult T-cell leukemia cells. Nutrition and cancer. 2005;52(2):189-201.

166. Cho TM, Kim WJ, Moon SK. AKT signaling is involved in fucoidan-induced inhibition of growth and migration of human bladder cancer cells. Food Chem Toxicol. 2014;64:344-52.

167. Kim EJ, Park SY, Lee JY, Park JH. Fucoidan present in brown algae induces apoptosis of human colon cancer cells. BMC gastroenterology. 2010;10.

168. Yamasaki-Miyamoto Y, Yamasaki M, Tachibana H, Yamada K. Fucoidan induces apoptosis through activation of caspase-8 on human breast cancer MCF-7 cells. J Agric Food Chem. 2009;57(18):8677-82.

169. Fukahori S, Yano H, Akiba J, Ogasawara S, Momosaki S, Sanada S, et al. Fucoidan, a major component of brown seaweed, prohibits the growth of human cancer cell lines in vitro. Mol Med Rep. 2008;1(4):537-42.

170. Jin JO, Song MG, Kim YN, Park JI, Kwak JY. The mechanism of fucoidan-induced apoptosis in leukemic cells: involvement of ERK1/2, JNK, glutathione, and nitric oxide. Mol Carcinog. 2010;49(8):771-82.

171. Boo H, Hyun J, Kim S, Kang J, Kim M, Kim S, et al. Fucoidan from Undaria pinnatifida Induces Apoptosis in A549 Human Lung Carcinoma Cells. Phytotherapy Research. 2011;25(7):1082-6.

172. Costa LS, Telles CB, Oliveira RM, Nobre LT, Dantas-Santos N, Camara RB, et al. Heterofucan from Sargassum filipendula induces apoptosis in HeLa cells. Marine drugs. 2011;9(4):603-14.

173. Yang L, Wang P, Wang H, Li Q, Teng H, Liu Z, et al. Fucoidan derived from Undaria pinnatifida induces apoptosis in human hepatocellular carcinoma SMMC-7721 cells via the ROS-mediated mitochondrial pathway. Marine drugs. 2013;11(6):1961-76.

174. Zhang Z, Teruya K, Eto H, Shirahata S. Induction of apoptosis by low-molecular-weight fucoidan through calcium- and caspase-dependent mitochondrial pathways in MDA-MB-231 breast cancer cells. Bioscience, biotechnology, and biochemistry. 2013;77(2):235-42.

175. Koyanagi S, Tanigawa N, Nakagawa H, Soeda S, Shimeno H. Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. Biochemical pharmacology. 2003;65(2):173-9.

176. Xue M, Ge Y, Zhang J, Wang Q, Hou L, Liu Y, et al. Anticancer properties and mechanisms of fucoidan on mouse breast cancer in vitro and in vivo. PloS one. 2012;7:e43483.

177. Coombe DR, Parish CR, Ramshaw IA, Snowden JM. Analysis of the inhibition of tumour metastasis by sulphated polysaccharides. International journal of cancer Journal international du cancer. 1987;39(1):82-8.

178. Soeda S, Ishida S, Shimeno H, Nagamatsu A. Inhibitory effect of oversulfated fucoidan on invasion through reconstituted basement membrane by murine Lewis lung carcinoma. Japanese journal of cancer research : Gann. 1994;85(11):1144-50.

179. Liu JM, Bignon J, Haroun-Bouhedja F, Bittoun P, Vassy J, Fermandjian S, et al. Inhibitory effect of fucoidan on the adhesion of adenocarcinoma cells to fibronectin. Anticancer research. 2005;25(3b):2129-33.

180. Lee H, Kim JS, Kim E. Fucoidan from seaweed Fucus vesiculosus inhibits migration and invasion of human lung cancer cell via PI3K-Akt-mTOR pathways. PloS one. 2012;7(11):e50624.

181. Cumashi A, Ushakova NA, Preobrazhenskaya ME, D'Incecco A, Piccoli A, Totani L, et al. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. Glycobiology. 2007;17(5):541-52.

182. Patel MK, Mulloy B, Gallagher KL, O'Brien L, Hughes AD. The antimitogenic action of the sulphated polysaccharide fucoidan differs from heparin in human vascular smooth muscle cells. Thrombosis and haemostasis. 2002;87(1):149-54.

183. Ale MT, Maruyama H, Tamauchi H, Mikkelsen JD, Meyer AS. Fucoidan from Sargassum sp. and Fucus vesiculosus reduces cell viability of lung carcinoma and melanoma cells in vitro and activates natural killer cells in mice in vivo. International journal of biological macromolecules. 2011;49(3):331-6.

184. Azuma K, Ishihara T, Nakamoto H, Amaha T, Osaki T, Tsuka T, et al. Effects of oral administration of fucoidan extracted from Cladosiphon okamuranus on tumor growth and survival time in a tumor-bearing mouse model. Marine drugs. 2012;10(10):2337-48.

185. Shimizu J, Wada-Funada1 U, Mano1 M, Matahira Y, Kawaguchi M, Wada M. Proportion of Murine Cytotoxic T Cells is Increased by High Molecular-Weight Fucoidan Extracted from Okinawa mozuku (Cladosiphon okamuranus). Journal of Health Science. 2005;51:394-7.

186. Hu Y, Cheng SC, Chan KT, Ke Y, Xue B, Sin FW, et al. Fucoidin enhances dendritic cell-mediated T-cell cytotoxicity against NY-ESO-1 expressing human cancer cells. Biochemical and biophysical research communications. 2010;392(3):329-34.

187. Jin JO, Zhang W, Du JY, Wong KW, Oda T, Yu Q. Fucoidan can function as an adjuvant in vivo to enhance dendritic cell maturation and function and promote antigen-specific T cell immune responses. PloS one. 2014;9(6):e99396.

188. Teas J, Harbison ML, Gelman RS. Dietary seaweed (Laminaria) and mammary carcinogenesis in rats. Cancer research. 1984;44(7):2758-61.

189. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. Current opinion in genetics & development. 2002;12(1):22-9.

190. Hsu HY, Lin TY, Hwang PA, Tseng LM, Chen RH, Tsao SM, et al. Fucoidan induces changes in the epithelial to mesenchymal transition and decreases metastasis by enhancing ubiquitin-dependent TGFbeta receptor degradation in breast cancer. Carcinogenesis. 2013;34(4):874-84.

191. Humphrey PA, Wong AJ, Vogelstein B, Zalutsky MR, Fuller GN, Archer GE, et al. Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. Proceedings of the National Academy of Sciences of the United States of America. 1990;87(11):4207-11.

192. Lee NY, Ermakova SP, Zvyagintseva TN, Kang KW, Dong Z, Choi HS. Inhibitory effects of fucoidan on activation of epidermal growth factor receptor and cell transformation in JB6 Cl41 cells. Food Chem Toxicol. 2008;46(5):1793-800.

193. Ikeguchi M, Yamamoto M, Arai Y, Maeta Y, Ashida K, Katano K, et al. Fucoidan reduces the toxicities of chemotherapy for patients with unresectable advanced or recurrent colorectal cancer. Oncology letters. 2011;2(2):319-22.

194. Lv J, Xiao Q, Wang L, Liu X, Wang X, Yang Z, et al. Fucoidan prevents multiple myeloma cell escape from chemotherapy-induced drug cytotoxicity. Fitoterapia. 2013;84:257-63.

195. Vishchuk OS, Ermakova SP, Zvyagintseva TN. The effect of sulfated (1-->3)-alpha-l-fucan from the brown alga Saccharina cichorioides Miyabe on resveratrol-induced apoptosis in colon carcinoma Cells. Marine drugs. 2013;11(1):194-212.

196. Zhang Z, Teruya K, Yoshida T, Eto H, Shirahata S. Fucoidan extract enhances the anti-cancer activity of chemotherapeutic agents in MDA-MB-231 and MCF-7 breast cancer cells. Marine drugs. 2013;11(1):81-98.

197. Oh B, Kim J. Anticancer effect of fucoidan in combination with tyrosine kinase inhibitor lapatinib. Evid Based Complement Alternat Med. 2014;2014:865375.

198. Collins SJ, Gallo RC, Gallagher RE. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature. 1977;270(5635):347-9.

199. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood. 1991;77(5):1080-6.

200. Andersson LC, Nilsson K, Gahmberg CG. K562--a human erythroleukemic cell line. International journal of cancer Journal international du cancer. 1979;23(2):143-7.

201. Koeffler HP, Golde DW. Acute myelogenous leukemia: a human cell line responsive to colony-stimulating activity. Science (New York, NY). 1978;200(4346):1153-4.

202. Asou H, Tashiro S, Hamamoto K, Otsuji A, Kita K, Kamada N. Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. Blood. 1991;77(9):2031-6.

203. Wright SC, Bonavida B. YAC-1 variant clones selected for resistance to natural killer cytotoxic factors are also resistant to natural killer cell-mediated cytotoxicity. Proceedings of the National Academy of Sciences of the United States of America. 1983;80(6):1688-92.

204. Castro J, Ribo M, Navarro S, Nogues MV, Vilanova M, Benito A. A human ribonuclease induces apoptosis associated with p21WAF1/CIP1 induction and JNK inactivation. BMC cancer. 2011;11:9-21.

205. Kanemura Y, Mori H, Kobayashi S, Islam O, Kodama E, Yamamoto A, et al. Evaluation of in vitro proliferative activity of human fetal neural stem/progenitor cells using indirect measurements of viable cells based on cellular metabolic activity. Journal of neuroscience research. 2002;69(6):869-79.

206. Schutte B, Nuydens R, Geerts H, Ramaekers F. Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. Journal of neuroscience methods. 1998;86(1):63-9.

207. Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic acids research. 1989;17(15):6419.

208. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976;72:248-54.

209. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric Method for Determination of Sugars and Related Substances. Analytical Chemistry,. 1956;28(3):350-6.

210. Dodgson KS. Determination of inorganic sulphate in studies on the enzymic and non-enzymic hydrolysis of carbohydrate and other sulphate esters. The Biochemical journal. 1961;78:312-9.

211. Dodgson KS, Price RG. A note on the determination of the ester sulphate content of sulphated polysaccharides. The Biochemical journal. 1962;84:106-10.

212. Bruce W, Zoecklein., Kenneth C, Fugelsang., Barry H, Gump., Fred S, Nury. Wine analysis and production: Springer Science+Business Media New York 1995.

213. Jiménez-Escrig A, Jiménez-Jiménez I, Pulido R, Saura-Calixto F. Antioxidant activity of fresh and processed edible seaweeds. J Sci Food Agr. 2001;81(5):530-4.

214. Zhang Q, Zhang J, Shen J, Silva A, Dennis D, A., Barrow C, J. A Simple 96-Well Microplate Method for Estimation of Total Polyphenol Content in Seaweeds. J Appl Phycol. 2006;18(3-5):445-50.

215. Morvai-Vitányi M, Molnár-Perl I, Knausz D, Sass P. Simultaneous GC derivatization and quantification of acids and sugars. Chromatographia. 1993;36(1):204-6.

216. American Veterinary Medical Association. AVMA guidelines on euthanasia, 2013 update [July 2015]. Available from: http://www.avma.org/issues/animal_welfare/euthanasia.pdf.

217. Roth V. Doubling time 2006 [10 Jan 2015]. Available from: http://www.doubling-time.com/compute.php.

218. Micallef IN, Rohatiner AZ, Carter M, Boyle M, Slater S, Amess JA, et al. Long-term outcome of patients surviving for more than ten years following treatment for acute leukaemia. Br J Haematol. 2001;113:443-5.

219. Senderowicz AM. Flavopiridol: the first cyclin-dependent kinase inhibitor in human clinical trials. Investigational new drugs. 1999;17(3):313-20.

220. Park HS, Kim GY, Nam TJ, Deuk Kim N, Hyun Choi Y. Antiproliferative activity of fucoidan was associated with the induction of apoptosis and autophagy in AGS human gastric cancer cells. Journal of food science. 2011;76(3):T77-83.

221. Atashrazm F, Lowenthal RM, Woods GM, Holloway AF, Karpiniec SS, Dickinson JL. Fucoidan Suppresses the Growth of Human Acute Promyelocytic Leukemia Cells In Vitro and In Vivo. Journal of cellular physiology. 2015.

222. U.S. Food and Drug Administration. Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers 2012 [10 Jan 2015]. Available from: http://www.fda.gov/drugs/guidancecomplianceregulatoryinformation/guidances/ucm314718.htm.

223. Rabinovitch P. Introduction to cell cycle analysis: Phoenix Flow Systems, Inc.; [cited 2015 27.4.15]. Available from: http://www.phnxflow.com/Introduction%20to%20Cell%20Cycle%20Analysis.pdf.

224. Ferreira AK, Meneguelo R, Pereira A, Mendonca Filho O, Chierice GO, Maria DA. Anticancer effects of synthetic phosphoethanolamine on Ehrlich ascites tumor: an experimental study. Anticancer research. 2012;32(1):95-104.

225. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Journal of immunological methods. 1995;184(1):39-51.

226. Hingorani R, Deng J, Elia J, McIntyre C, Mittar D. Detection of Apoptosis Using the BD Annexin V FITC Assay on the BD FACSVerse™ System. In: Biosciences. B, editor. BD Biosciences2011. p. 2.

227. Haroun-Bouhedja F, Ellouali M, Sinquin C, Boisson-Vidal C. Relationship between sulfate groups and biological activities of fucans. Thrombosis research. 2000;100(5):453-9.

228. Ale MT, Mikkelsen JD, Meyer AS. Important determinants for fucoidan bioactivity: a critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweeds. Marine drugs. 2011;9(10):2106-30.

229. Cho ML, Lee BY, You SG. Relationship between oversulfation and conformation of low and high molecular weight fucoidans and evaluation of their in vitro anticancer activity. Molecules (Basel, Switzerland). 2010;16(1):291-7.

230. Codee JD, Christina AE, Walvoort MT, Overkleeft HS, van der Marel GA. Uronic acids in oligosaccharide and glycoconjugate synthesis. Topics in current chemistry. 2011;301:253-89.

231. Garron ML, Cygler M. Structural and mechanistic classification of uronic acid-containing polysaccharide lyases. Glycobiology. 2010;20(12):1547-73.

232. Rosenberg RD, Lam L. Correlation between structure and function of heparin. Proceedings of the National Academy of Sciences of the United States of America. 1979;76(3):1218-22.

233. Adibekian A, Bindschadler P, Timmer MS, Noti C, Schutzenmeister N, Seeberger PH. De novo synthesis of uronic acid building blocks for assembly of heparin oligosaccharides. Chemistry (Weinheim an der Bergstrasse, Germany). 2007;13(16):4510-22.

234. Rosenberg RD, Armand G, Lam L. Structure-function relationships of heparin species. Proceedings of the National Academy of Sciences of the United States of America. 1978;75(7):3065-9.

235. Giraux JL, Tapon-Bretaudiere J, Matou S, Fischer AM. Fucoidan, as heparin, induces tissue factor pathway inhibitor release from cultured human endothelial cells. Thrombosis and haemostasis. 1998;80(4):692-5.

236. Zhuang C, Itoh H, Mizuno T, Ito H. Antitumor active fucoidan from the brown seaweed, umitoranoo (Sargassum thunbergii). Bioscience, biotechnology, and biochemistry. 1995;59(4):563-7.

237. Vijayabaskar P, Shiyamala V. Antioxidant properties of seaweed polyphenol from Turbinaria ornata (Turner) J. Agardh, 1848. Asian Pac J Trop Biomed. 2012;2(1).

238. Souza AC, Machado FS, Celes MR, Faria G, Rocha LB, Silva JS, et al. Mitochondrial damage as an early event of monensin-induced cell injury in cultured fibroblasts L929. Journal of veterinary medicine A, Physiology, pathology, clinical medicine. 2005;52(5):230-7.

239. Park WH, Lee MS, Park K, Kim ES, Kim BK, Lee YY. Monensin-mediated growth inhibition in acute myelogenous leukemia cells via cell cycle arrest and apoptosis. International journal of cancer Journal international du cancer. 2002;101(3):235-42.

240. Lee HE, Choi ES, Shin JA, Lee SO, Park KS, Cho NP, et al. Fucoidan induces caspase-dependent apoptosis in MC3 human mucoepidermoid carcinoma cells. Experimental and therapeutic medicine. 2014;7(1):228-32.

241. Gillham CM, Reynolds J, Hollywood D. Predicting the response of localised oesophageal cancer to neo-adjuvant chemoradiation. World journal of surgical oncology. 2007;5:97.

242. Hyun JH, Kim SC, Kang JI, Kim MK, Boo HJ, Kwon JM, et al. Apoptosis inducing activity of fucoidan in HCT-15 colon carcinoma cells. Biol Pharm Bull. 2009;32(10):1760-4.

243. Osaki M, Oshimura M, Ito H. PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis : an international journal on programmed cell death. 2004;9:667-76.

244. Gallay N, Dos Santos C, Cuzin L, Bousquet M, Simmonet Gouy V, Chaussade C, et al. The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. Leukemia. 2009;23(6):1029-38.

245. Zhuang S, Schnellmann RG. A death-promoting role for extracellular signal-regulated kinase. The Journal of pharmacology and experimental therapeutics. 2006;319(3):991-7.

246. Xiao Y, Zou P, Wang J, Song H, Zou J, Liu L. Lower phosphorylation of p38 MAPK blocks the oxidative stress-induced senescence in myeloid leukemic CD34(+)CD38 (-) cells. J Huazhong Univ Sci Technolog Med Sci. 2012;32(3):328-33.

247. Park HS, Hwang HJ, Kim GY, Cha HJ, Kim WJ, Kim ND, et al. Induction of apoptosis by fucoidan in human leukemia U937 cells through activation of p38 MAPK and modulation of Bcl-2 family. Marine drugs. 2013;11(7):2347-64.

248. Namvar F, Mohamad R, Baharara J, Zafar-Balanejad S, Fargahi F, Rahman HS. Antioxidant, antiproliferative, and antiangiogenesis effects of polyphenol-rich seaweed (Sargassum muticum). BioMed research international. 2013;2013:604787.

249. Mak W, Wang SK, Liu T, Hamid N, Li Y, Lu J, et al. Anti-Proliferation Potential and Content of Fucoidan Extracted from Sporophyll of New Zealand Undaria pinnatifida. Frontiers in nutrition. 2014;1:9.

250. Miao HQ, Elkin M, Aingorn E, Ishai-Michaeli R, Stein CA, Vlodavsky I. Inhibition of heparanase activity and tumor metastasis by laminarin sulfate and synthetic phosphorothioate

oligodeoxynucleotides. International journal of cancer Journal international du cancer. 1999;83(3):424-31.

251. Kotake-Nara E, Sugawara T, Nagao A. Antiproliferative effect of neoxanthin and fucoxanthin on cultured cells. Fisheries Science. 2005;71(2):459-61.

252. Reddy BS, Cohen LA, McCoy GD, Hill P, Weisburger JH, Wynder EL. Nutrition and its relationship to cancer. Advances in cancer research. 1980;32:237-345.

253. Teas J, Vena S, Cone DL, Irhimeh M. The consumption of seaweed as a protective factor in the etiology of breast cancer: proof of principle. Journal of applied phycology. 2013;25(3):771-9.

254. Kotzsch M, Bernt K, Friedrich K, Luther E, Albrecht S, Gatzweiler A, et al. Prognostic relevance of tumour cell-associated uPAR expression in invasive ductal breast carcinoma. Histopathology. 2010;57(3):461-71.

255. Furusawa E, Furusawa S, Chou SC. Antileukemic activity of Viva-Natural, a dietary seaweed extract, on Rauscher murine leukemia in comparison with anti-HIV agents, azidothymidine, dextran sulfate and pentosan polysulfate. Cancer letters. 1991;56(3):197-205.

256. Harada H, Kamei Y. Selective cytotoxicity of marine algae extracts to several human leukemic cell lines. Cytotechnology. 1997;25(1-3):213-9.

257. Goldstein BD, Witz G. Free radicals and carcinogenesis. Free radical research communications. 1990;11(1-3):3-10.

258. Ahmed S, Othman NH. Honey as a potential natural anticancer agent: a review of its mechanisms. Evidence-based complementary and alternative medicine : eCAM. 2013;2013:829070.

259. Rocha de Souza MC, Marques CT, Guerra Dore CM, Ferreira da Silva FR, Oliveira Rocha HA, Leite EL. Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. Journal of applied phycology. 2007;19(2):153-60.

260. Omar HM, Saadeldin HM, Badary MS, Al-khatib BY, Abdelgaffar SK. The immunomodulating and antioxidant activity of fucoidan on the splenic tissue of rats treated with cyclosporine A. The Journal of Basic & Applied Zoology. 2013;66(5):243-54.

261. Maruyama H, Tamauchi H, Iizuka M, Nakano T. The role of NK cells in antitumor activity of dietary fucoidan from Undaria pinnatifida sporophylls (Mekabu). Planta medica. 2006;72(15):1415-7.

262. Furusawa E, Furusawa S. Anticancer activity of a natural product, viva-natural, extracted from Undaria pinnantifida on intraperitoneally implanted Lewis lung carcinoma. Oncology. 1985;42(6):364-9.

263. Yang M, Ma C, Sun J, Shao Q, Gao W, Zhang Y, et al. Fucoidan stimulation induces a functional maturation of human monocyte-derived dendritic cells. International immunopharmacology. 2008;8(13-14):1754-60.

264. Zhang W, Oda T, Yu Q, Jin JO. Fucoidan from Macrocystis pyrifera has powerful immune-modulatory effects compared to three other fucoidans. Marine drugs. 2015;13(3):1084-104.

265. Michikawa T, Inoue M, Shimazu T, Sawada N, Iwasaki M, Sasazuki S, et al. Seaweed consumption and the risk of thyroid cancer in women: the Japan Public Health Center-based Prospective Study. European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP). 2012;21(3):254-60.

266. Huang TH, Chiu YH, Chan YL, Chiu YH, Wang H, Huang KC, et al. Prophylactic administration of fucoidan represses cancer metastasis by inhibiting vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) in Lewis tumor-bearing mice. Marine drugs. 2015;13(4):1882-900.

267. An JJ, Shi KJ, Wei W, Hua FY, Ci YL, Jiang Q, et al. The ROS/JNK/ATF2 pathway mediates selenite-induced leukemia NB4 cell cycle arrest and apoptosis in vitro and in vivo. Cell death & disease. 2013;4:e973.

268. Hampson P, Wang K, Ersvaer E, McCormack E, Schuler J, Fiebig HH, et al. Up-regulation of anti-apoptotic genes confers resistance to the novel anti-leukaemic compound PEP005 in primary AML cells. Oncoscience. 2014;1(8):529-39.

269. Zhang SY, Zhu J, Chen GQ, Du XX, Lu LJ, Zhang Z, et al. Establishment of a human acute promyelocytic leukemia-ascites model in SCID mice. Blood. 1996;87(8):3404-9.

270. Zuo T, Li X, Chang Y, Duan G, Yu L, Zheng R, et al. Dietary fucoidan of Acaudina molpadioides and its enzymatically degraded fragments could prevent intestinal mucositis induced by chemotherapy in mice. Food & function. 2015;6(2):415-22.

271. Maruyama H, Tamauchi H, Hashimoto M, Nakano T. Antitumor activity and immune response of Mekabu fucoidan extracted from Sporophyll of Undaria pinnatifida. In Vivo. 2003;17(3):245-9.

272. Tallman MS, Altman JK. How I treat acute promyelocytic leukemia. Blood. 2009;114(25):5126-35.

273. Patatanian E, Thompson DF. Retinoic acid syndrome: a review. Journal of clinical pharmacy and therapeutics. 2008;33(4):331-8.

274. Specchia G, Lo Coco F, Vignetti M, Avvisati G, Fazi P, Albano F, et al. Extramedullary involvement at relapse in acute promyelocytic leukemia patients treated or not with all-trans retinoic acid: a report by the Gruppo Italiano Malattie Ematologiche dell'Adulto. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2001;19(20):4023-8.

275. Lobe I, Rigal-Huguet F, Vekhoff A, Desablens B, Bordessoule D, Mounier C, et al. Myelodysplastic syndrome after acute promyelocytic leukemia: the European APL group experience. Leukemia. 2003;17(8):1600-4.

276. Thomas X, Le QH, Fiere D. Anthracycline-related toxicity requiring cardiac transplantation in long-term disease-free survivors with acute promyelocytic leukemia. Annals of hematology. 2002;81(9):504-7.

277. Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY, et al. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood. 1997;89(9):3354-60.

278. Soignet SL, Maslak P, Wang ZG, Jhanwar S, Calleja E, Dardashti LJ, et al. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. The New England journal of medicine. 1998;339(19):1341-8.

279. Miller WH, Jr., Schipper HM, Lee JS, Singer J, Waxman S. Mechanisms of action of arsenic trioxide. Cancer research. 2002;62(14):3893-903.

280. Zhang TD, Chen GQ, Wang ZG, Wang ZY, Chen SJ, Chen Z. Arsenic trioxide, a therapeutic agent for APL. Oncogene. 2001;20(49):7146-53.

281. Calleja EM, Warrell RP, Jr. Differentiating agents in pediatric malignancies: all-trans-retinoic acid and arsenic in acute promyelocytic leukemia. Current oncology reports. 2000;2(6):519-23.

282. Niu C, Yan H, Yu T, Sun HP, Liu JX, Li XS, et al. Studies on treatment of acute promyelocytic leukemia with arsenic trioxide: remission induction, follow-up, and molecular monitoring in 11 newly diagnosed and 47 relapsed acute promyelocytic leukemia patients. Blood. 1999;94(10):3315-24.

283. Zhou GB, Zhang J, Wang ZY, Chen SJ, Chen Z. Treatment of acute promyelocytic leukaemia with all-trans retinoic acid and arsenic trioxide: a paradigm of synergistic molecular targeting therapy. Philos Trans R Soc Lond B Biol Sci. 2007;362(1482):959-71.

284. Alekseyenko TV, Zhanayeva SY, Venediktova AA, Zvyagintseva TN, Kuznetsova TA, Besednova NN, et al. Antitumor and antimetastatic activity of fucoidan, a sulfated polysaccharide isolated from the Okhotsk Sea Fucus evanescens brown alga. Bulletin of experimental biology and medicine. 2007;143(6):730-2.

285. Kang YH, Yi MJ, Kim MJ, Park MT, Bae S, Kang CM, et al. Caspase-independent cell death by arsenic trioxide in human cervical cancer cells: reactive oxygen species-mediated poly(ADP-ribose) polymerase-1 activation signals apoptosis-inducing factor release from mitochondria. Cancer research. 2004;64(24):8960-7.

286. Petrie K, Zelent A, Waxman S. Differentiation therapy of acute myeloid leukemia: past, present and future. Curr Opin Hematol. 2009;16(2):84-91.

287. Lu KH, Lee HJ, Huang ML, Lai SC, Ho YL, Chang YS, et al. Synergistic Apoptosis-Inducing Antileukemic Effects of Arsenic Trioxide and Mucuna macrocarpa Stem Extract in Human Leukemic Cells via a Reactive Oxygen Species-Dependent Mechanism. Evidence-based complementary and alternative medicine : eCAM. 2012;2012:921430-44.

288. Fan Y, Chen M, Meng J, Yu L, Tu Y, Wan L, et al. Arsenic trioxide and resveratrol show synergistic anti-leukemia activity and neutralized cardiotoxicity. PloS one. 2014;9(8):e105890.

289. Wang Z, Zhang H, Dai L, Song T, Li P, Liu Y, et al. Arsenic Trioxide and Icariin Show Synergistic Anti-leukemic Activity. Cell biochemistry and biophysics. 2015.

290. Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. Oncogene. 2005;24(50):7455-64.

291. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. CA: a cancer journal for clinicians. 2002;52(1):23-47.

292. Mann KK, Colombo M, Miller WH, Jr. Arsenic trioxide decreases AKT protein in a caspase-dependent manner. Molecular cancer therapeutics. 2008;7(6):1680-7.

293. Scholz C, Wieder T, Starck L, Essmann F, Schulze-Osthoff K, Dorken B, et al. Arsenic trioxide triggers a regulated form of caspase-independent necrotic cell death via the mitochondrial death pathway. Oncogene. 2005;24(11):1904-13.

294. Xie J, Zhang M, Song YP, Hu K, Ren JJ, Zhang YJ. [Caspase-independent apoptosis induced by arsenic trioxide in human multiple myeloma cell RPMI8226]. Zhongguo shi yan xue ye xue za zhi / Zhongguo bing li sheng li xue hui = Journal of experimental hematology / Chinese Association of Pathophysiology. 2012;20(1):107-11.

295. Chaitanya GV, Steven AJ, Babu PP. PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. Cell communication and signaling : CCS. 2010;8:31.

296. Lee J, Kim J, Moon C, Kim SH, Hyun JW, Park JW, et al. Radioprotective effects of fucoidan in mice treated with total body irradiation. Phytotherapy research : PTR. 2008;22(12):1677-81.

297. Prebet T, Gore SD. Treatment of acute promyelocytic leukemia for older patients. Journal of the National Comprehensive Cancer Network : JNCCN. 2011;9(3):337-42.

298. de The H, Le Bras M, Lallemand-Breitenbach V. The cell biology of disease: Acute promyelocytic leukemia, arsenic, and PML bodies. The Journal of cell biology. 2012;198(1):11-21.

299. Martin SJ, Bradley JG, Cotter TG. HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. Clin Exp Immunol. 1990;79(3):448-53.

300. Yoshida H, Kitamura K, Tanaka K, Omura S, Miyazaki T, Hachiya T, et al. Accelerated degradation of PML-retinoic acid receptor alpha (PML-RARA) oncoprotein by all-trans-

retinoic acid in acute promyelocytic leukemia: possible role of the proteasome pathway. Cancer research. 1996;56(13):2945-8.

301. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, et al. Steroid receptor coactivator-1 is a histone acetyltransferase. Nature. 1997;389(6647):194-8.

302. Guidez F, Ivins S, Zhu J, Soderstrom M, Waxman S, Zelent A. Reduced retinoic acid-sensitivities of nuclear receptor corepressor binding to PML- and PLZF-RARalpha underlie molecular pathogenesis and treatment of acute promyelocytic leukemia. Blood. 1998;91(8):2634-42.

303. Zhong S, Muller S, Ronchetti S, Freemont PS, Dejean A, Pandolfi PP. Role of SUMO-1-modified PML in nuclear body formation. Blood. 2000;95(9):2748-52.

304. Zhu J, Lallemand-Breitenbach V, de The H. Pathways of retinoic acid- or arsenic trioxide-induced PML/RARalpha catabolism, role of oncogene degradation in disease remission. Oncogene. 2001;20(49):7257-65.

305. Ying M, Zhou X, Zhong L, Lin N, Jing H, Luo P, et al. Bortezomib sensitizes human acute myeloid leukemia cells to all-trans-retinoic acid-induced differentiation by modifying the RARalpha/STAT1 axis. Molecular cancer therapeutics. 2013;12(2):195-206.

306. Adamson PC. All-Trans-Retinoic Acid Pharmacology and Its Impact on the Treatment of Acute Promyelocytic Leukemia. The oncologist. 1996;1(5):305-14.

307. Prudovsky I, Popov K, Akimov S, Serov S, Zelenin A, Meinhardt G, et al. Antisense CD11b integrin inhibits the development of a differentiated monocyte/macrophage phenotype in human leukemia cells. European journal of cell biology. 2002;81(1):36-42.

308. Estey E, Garcia-Manero G, Ferrajoli A, Faderl S, Verstovsek S, Jones D, et al. Use of all-trans retinoic acid plus arsenic trioxide as an alternative to chemotherapy in untreated acute promyelocytic leukemia. Blood. 2006;107(9):3469-73.

309. Camacho LH, Soignet SL, Chanel S, Ho R, Heller G, Scheinberg DA, et al. Leukocytosis and the retinoic acid syndrome in patients with acute promyelocytic leukemia treated with arsenic trioxide. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2000;18(13):2620-5.

310. Guo XH, Yasen H, Jiang M, Hao JP, Abulaiti D, Chen R. [Retinoic acid in treating acute promyelocytic leukemia with hyperleukocytosis and its therapeutic strategy]. Zhongguo shi yan xue ye xue za zhi / Zhongguo bing li sheng li xue hui = Journal of experimental hematology / Chinese Association of Pathophysiology. 2008;16(2):439-41.

311. Chen GQ, Shi XG, Tang W, Xiong SM, Zhu J, Cai X, et al. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): I. As2O3 exerts dose-dependent dual effects on APL cells. Blood. 1997;89(9):3345-53.

312. Solovjov DA, Pluskota E, Plow EF. Distinct roles for the alpha and beta subunits in the functions of integrin alphaMbeta2. The Journal of biological chemistry. 2005;280(2):1336-45.

313. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science (New York, NY). 1988;241(4861):58-62.

314. Yang L, Zhao H, Li SW, Ahrens K, Collins C, Eckenrode S, et al. Gene expression profiling during all-trans retinoic acid-induced cell differentiation of acute promyelocytic leukemia cells. The Journal of molecular diagnostics : JMD. 2003;5(4):212-21.

315. Kinjo K, Kizaki M, Muto A, Fukuchi Y, Umezawa A, Yamato K, et al. Arsenic trioxide (As2O3)-induced apoptosis and differentiation in retinoic acid-resistant acute promyelocytic leukemia model in hGM-CSF-producing transgenic SCID mice. Leukemia. 2000;14(3):431-8.

316. Kolly C, Suter MM, Muller EJ. Proliferation, cell cycle exit, and onset of terminal differentiation in cultured keratinocytes: pre-programmed pathways in control of C-Myc and Notch1 prevail over extracellular calcium signals. The Journal of investigative dermatology. 2005;124(5):1014-25.

317. Dos Santos GA, Kats L, Pandolfi PP. Synergy against PML-RARa: targeting transcription, proteolysis, differentiation, and self-renewal in acute promyelocytic leukemia. The Journal of experimental medicine. 2013;210(13):2793-802.

318. Kim BS, Kang HJ, Park JY, Lee J. Fucoidan promotes osteoblast differentiation via JNK- and ERK-dependent BMP2-Smad 1/5/8 signaling in human mesenchymal stem cells. Experimental & molecular medicine. 2015;47:e128.

319. Coffer PJ, Koenderman L, de Groot RP. The role of STATs in myeloid differentiation and leukemia. Oncogene. 2000;19(21):2511-22.

320. Byon YY, Kim MH, Yoo ES, Hwang KK, Jee Y, Shin T, et al. Radioprotective effects of fucoidan on bone marrow cells: improvement of the cell survival and immunoreactivity. Journal of veterinary science. 2008;9(4):359-65.

321. Irhimeh MR, Fitton JH, Lowenthal RM. Fucoidan ingestion increases the expression of CXCR4 on human CD34+ cells. Experimental hematology. 2007;35(6):989-94.

322. Nakajima H, Ihle JN. Granulocyte colony-stimulating factor regulates myeloid differentiation through CCAAT/enhancer-binding protein epsilon. Blood. 2001;98(4):897-905.

323. Liu J, Bi G, Wen P, Yang W, Ren X, Tang T, et al. Down-regulation of CD44 contributes to the differentiation of HL-60 cells induced by ATRA or HMBA. Cellular & molecular immunology. 2007;4(1):59-63.

324. Elliott VA, Rychahou P, Zaytseva YY, Evers BM. Activation of c-Met and upregulation of CD44 expression are associated with the metastatic phenotype in the colorectal cancer liver metastasis model. PloS one. 2014;9(5):e97432.

325. Rego EM, De Santis GC. Differentiation syndrome in promyelocytic leukemia: clinical presentation, pathogenesis and treatment. Mediterranean journal of hematology and infectious diseases. 2011;3(1):e2011048.

326. Crawford S. Is it time for a new paradigm for systemic cancer treatment? Lessons from a century of cancer chemotherapy. Frontiers in pharmacology. 2013;4:68.

327. Burger JA. Nurture versus nature: the microenvironment in chronic lymphocytic leukemia. Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program. 2011;2011:96-103.

328. Lucas DM, Still PC, Perez LB, Grever MR, Kinghorn AD. Potential of plant-derived natural products in the treatment of leukemia and lymphoma. Current drug targets. 2010;11(7):812-22.

329. Donoghue M, Lemery SJ, Yuan W, He K, Sridhara R, Shord S, et al. Eribulin mesylate for the treatment of patients with refractory metastatic breast cancer: use of a "physician's choice" control arm in a randomized approval trial. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012;18(6):1496-505.

330. Wang P, Liu Z, Liu X, Teng H, Zhang C, Hou L, et al. Anti-metastasis effect of fucoidan from Undaria pinnatifida sporophylls in mouse hepatocarcinoma Hca-F cells. PloS one. 2014;9(8):e106071.

331. Choosawad D, Leggat U, Dechsukhum C, Phongdara A, Chotigeat W. Antitumour activities of fucoidan from the aquatic plant Utricularia aurea lour. J Sci Technol. 2005;27 (Suppl. 3):799-807.

332. Yang H, Zeng M, Dong S, Liu Z, Li R. Anti-proliferative activity of phlorotannin extracts from brown algae Laminaria japonica Aresch. Chinese Journal of Oceanology and Limnology. 2010;28(1):122-30.

333. Cooper R, Dragar C, Elliot K, Fitton JH, Godwin J, Thompson K. GFS, a preparation of Tasmanian Undaria pinnatifida is associated with healing and inhibition of reactivation of Herpes. BMC complementary and alternative medicine. 2002;2:11.

334. Chun YJ, Park IC, Park MJ, Woo SH, Hong SI, Chung HY, et al. Enhancement of radiation response in human cervical cancer cells in vitro and in vivo by arsenic trioxide (As2O3). FEBS letters. 2002;519(1-3):195-200.

335. Du Y, Wang K, Fang H, Li J, Xiao D, Zheng P, et al. Coordination of intrinsic, extrinsic, and endoplasmic reticulum-mediated apoptosis by imatinib mesylate combined with arsenic trioxide in chronic myeloid leukemia. Blood. 2006;107(4):1582-90.

Appendix A- Reagents

Reagent	Preparation
1M NaCl	5.84 gr NaCl powder was dissolved in 100 mL Milli-Q water and stored at 2-8°C.
1 M Tris	12.114 gr Tris powder was dissolved in 100 mL Milli-Q water and stored at 2-8°C.
1 M $MgCl_2$	9.52 gr $MgCl_2$ powder was dissolved in 100 mL Milli-Q water and stored at 2-8°C.
0.5 M EDTA	1.46 gr EDTA powder was dissolved in 10 mL Milli-Q water and stored at 2-8°C.
0.5 M Tris HCl, pH 6.8	12.1 gr of Tris base was dissolved in 150 mL of Milli-Q water water. pH was adjusted to 6.8 using concentrated HCl and the total volume to 200 mL was made up with Milli-Q water. The solution was stored at 2-8 °C.
1.65 M NaOH	66 mg NaOH powder was dissolved in 1 mL Milli-Q water.
protease inhibitor cocktail	Complete protease inhibitor cocktail tablets were purchased from Roche Co. (Cat. No. 04693116001). Each tablet was dissolved in 2 mL Milli-Q water and aliquots were stored in -20 °C freezer.
10% Igepal	The Igepal solution was purchased from Sigma-Aldrich, U.S.A. To make the 10% Igepal, 2 mL of the stock solution was diluted in 18 mL Milli-Q water and was stored at 2-8 °C.

Primary and secondary antibodies utilised for Western blot

Affinity	MW (KD)	Host	Dilution*	Supplier (Cat. No.)
Anti-Pro Caspase 3	32	Rabbit	1:100	Abcam (ab136812)
Anti-Caspase 3	17	Rabbit	1:100	Abcam (ab136812)
Anti-PARP	89	Rabbit	1:100	Abcam (ab136812)
Anti-Pro Caspase 8	55	Goat	1:200	Santa Cruz (sc-6134)
Anti- Caspase 8	18	Goat	1:200	Santa Cruz (sc-6134)
Anti-Pro Caspase 9	46	Rabbit	1:200	Santa Cruz (sc-8355)
Anti- Caspase 9	35	Rabbit	1:200	Santa Cruz (sc-8355)
Anti-ERK	42,44	Rabbit	1:1000	Cell Signalling (4695P)
Anti-p-ERK	42,44	Rabbit	1:2000	Cell Signalling (4370P)
Anti-AKT	60	Rabbit	1:1000	Cell Signalling (4691P)
Anti-p-AKT(Ser)	60	Rabbit	1:2000	Cell Signalling (4060P)
Anti-p-AKT(Thr)	60	Rabbit	1:1000	Cell Signalling (2965P)
Anti-Bcl-xl	26,30	Rabbit	1:1000	Cell Signalling (2764P)
Anti-DR5	40,48	Rabbit	1:1000	Cell Signalling (8074P)
Anti-Fas	40-50	Rabbit	1:1000	Cell Signalling (4233S)
Anti-Bax	21	Rabbit	1:1000	Cell Signalling (5023P)
Anti-Cyclin D1	36	Mouse	1:2000	Cell Signalling (2926P)
Anti-Waf1/Cip1	21	Mouse	1:2000	Cell Signalling (2946P)
Anti-Actin	42	Rabbit	1:1000	Abcam (ab184111)
Anti-GAPDH	36	Mouse	1:5000	Millipore (MAB374)
Anti-SP1	90	Rabbit	1:1000	Santa Cruz (sc-59)
Secondary Antibodies Labelled with HRP				
Anti-Rabbit/Mouse**		Goat	1:250	Abcam (ab136812)
Anti-Rabbit		Goat	1:1000	Dako (P0448)
Anti-Goat		Rabbit	1:1000	Dako (P0160)
Anti-Mouse		Rabbit	1:1000	Dako (P0260)

* Various antibody dilutions were diluted in 10 mL of 1X TNT with 1% 10X blocking reagent.
**: Cocktail secondary antibody for anti-caspase 3 and anti-PARP was provided by the supplier in the kit

Appendix B- Methods

Quantitation of Protein by Bradford Assay

Bradford dye reagent was purchased from Bio-Rad, U.S.A and stored in the fridge. To measure the proteins concentrations, the dye reagent was diluted 1:5 with Milli-Q water. The extracts were diluted 1:10 with Milli-Q water and serial dilutions of bovine serum albumin (BSA) were used to further standard curve construction (i.e. 0.1 mg/mL, 0.15 mg/mL, 0.2 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL samples of BSA were prepared from 10 mg/mL, BSA; Invitrogen™, U.S.A). 990 µL of the diluted Bradford dye reagent was added in 10 µL of each extract and each diluted BSA standard. Absorbance was measured at 595 nm using the SpectraMax® Plus384 Absorbance Reader (Molecular Devices, U.S.A). The results were entered in Microsoft Excel 2010 and BSA standard curve was created. Sample protein concentrations were determined in comparison to the BSA standard curve.

Appendix C –Monitoring Sheets

Animal monitoring sheet after tumour cells injection

Project code: A13940					
Aim	**Mouse Ear tag- ID**	**Cage**	**Tumour injection date**	**Treatment start**	**Termination date**
…….	……..	…….	……	……..	……

Tumour Appearance Monitoring			
Day	**Date**	**Weight**	**Condition/comment**
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			

Animal monitoring sheets during Treatment

Project code: A13940					
Aim	**Mouse Ear tag- ID**	**Cage**	**Tumour injection date**	**Treatment start**	**Termination date**
…….	……..	…….	……	……..	……

Tumour Development				
Day	**Date**	**Weight**	**Tumour measurement Width x Length (Volume)**	**Condition/comment**
1			× ( )	
2			× ( )	
3			× ( )	
4			× ( )	
5			× ( )	
6			× ( )	
7			× ( )	
8			× ( )	
9			× ( )	
10			× ( )	
11			× ( )	
12			× ( )	
13			× ( )	
14			× ( )	
15			× ( )	
16			× ( )	
17			× ( )	
20			× ( )	
21			× ( )	
22			× ( )	
23			× ( )	
24			× ( )	
25			× ( )	
26			× ( )	
27			× ( )	
28			× ( )	

Appendix D-Results

Chapter 4. Daily body weight in mice fed with peanut butter and vehicle (control group)

Day before tumour injection	Control 1	Control 2	Control 3	Control 4	Control 5	Control 6	Control 7
1	12	12.1	21.52	18.75	22.45	12.3	23.6
2	12.78	12	21.3	18.83	22.36	12.81	23.84
3	13.36	12.25	21.88	18.73	23.07	12.95	24.44
4	13.5	12.48	21.93	19.13	23.11	13.25	24.6
5	13.7	12.26	21.88	19.66	23.23	12.99	24.7
6	13.75	12.2	21.54	19.55	23.04	12.99	23.84
7	13.84	12.6	21.6	19.5	23.5	13.25	24.23
8	14.01	12.95	21.8	19.91	23.1	13.6	24.65
9	13.48	12.6	21.71	19.23	22.9	13.32	24.14
10	13.57	12.8	21.64	19.31	22.6	13.7	23.85
11	14.15	13.22	21.33	19.73	22.81	14.12	23.06
12	14.54	13.49	21.47	19.81	22.9	14.61	23.19
13	14.73	13.45	21.56	19.8	22.76	14.43	23.32
14	15.04	13.2	21.8	19.72	22.83	14.8	23.6
Day after tumour injection							
1	15.3	13.33	21.64	19.73	22.6	14.81	24.21
2	15.68	13.71	21.61	20.06	22.81	14.94	24.26
3	15.66	13.6	21.35	20.22	22.7	14.9	24.57
4	15.8	13.9	21.88	20.34	23.28	15.54	24.48
5	16.1	14.01	21.84	20.68	23.7	15.03	24.17
6	16.3	14.15	22.36	21.4	24.02	15.42	24.64
7	15.91	14.32	22.13	21.28	23.31	15.46	24.4
8	16.5	14.2	22.4	21.1	23.71	15.14	24.81
9	16.23	14.8	22.14	21.65	23.73	15.6	24.54
10	16.3	14.65	23.61	21.74	24.86	16.16	25.45
11	15.98	14.8	22.6	21.29	24.5	16.35	25.4
12	16.5	15	22.5	20.85	24.7	16.38	25.8
13	16.14	15.32	22.36	20.82	24.07	16.92	24.49
14	16.26	15.1	22.4	20.73	24.5	17.41	25
15	16.62	15.26	22.91	21.55	24.61	17.78	25.25
16	16.58	15.41	23.11	21.68	**End Point**	17.86	25.29
17	17.11	15.48	22.97	21.2		**End Point**	24.42
18	17.47	15.61	**End Point**	**End Point**			25.8
19	**End Point**	**End Point**					24.9
20							24.69
21							25.25
22							25.8
23							25.4
24							**End Point**

Chapter 4. Daily body weight in mice fed with peanut butter and fucoidan (fucoidan group)

Day before tumour injection	Fucoidan 1	Fucoidan 2	Fucoidan 3	Fucoidan 4	Fucoidan 5	Fucoidan 6	Fucoidan 7
1	11.87	14.9	23.13	13	23.74	11.8	21.99
2	12.1	15.45	23.52	13.28	24.18	12.2	21.95
3	12.6	15.7	23.4	13.6	14.32	12.44	22.68
4	12.71	16	23.5	13.9	24.51	12.7	22.69
5	13	16.2	23.4	14.01	24.75	13.03	22.85
6	13.4	16.35	23.99	14.27	24	13.29	22.4
7	13.5	16.09	23.6	14	24.32	13.09	22.76
8	13.67	15.9	24.13	13.98	24.65	13.35	23
9	13.31	15.8	24.03	13.73	24.01	12.86	23.08
10	13.34	16.04	24.11	13.74	23.29	12.9	23.14
11	13.7	16.23	24.2	14.2	23.9	13.4	23.39
12	14.01	16.56	24.15	14.56	23.47	13.63	23
13	14.01	16.03	24.23	14.3	23.5	13.23	23.1
14	14.12	16.46	24.1	14.65	23.81	14.57	22.8
Day after tumour injection							
1	14.1	16.31	24.4	14.01	24.41	14.3	23.4
2	14	16.13	24.8	14.27	24.42	13.5	23.62
3	14.25	16.44	24.93	14	23.81	13.7	23.4
4	14.38	16.47	24.31	13.98	24.34	13.66	23.6
5	14.72	16.93	24.47	13.73	24.1	14.28	23.6
6	14.33	16.98	24.36	13.74	24.68	14.67	23.9
7	14.36	17.23	23.83	14.2	25.05	14.8	23.81
8	14.69	17.56	23.87	14.56	24.78	14.96	23.91
9	14.7	17.48	23.96	14.46	24.79	14.9	24.11
10	15.12	17.45	24.26	14.53	24.26	15.23	23.91
11	15.33	17.8	23.7	14.78	25	15.22	24.21
12	14.89	18.1	23.69	14.6	25.4	15.54	23.96
13	15.26	18	23.5	14.9	25.2	15.36	24.48
14	15.6	18.32	23.17	14.52	25.3	15.72	23.97
15	15.88	18.4	23.59	14.31	24.75	15.78	24.43
16	15.81	18.68	23.02	14.5	25.07	15.73	24.15
17	16.38	19.1	23.91	14.52	25.01	15.78	24.1
18	16.11	20.1	**End Point**	14.95	25.1	15.91	23.92
19	16.42	20.36		15.2	24.72	15.9	23.75
20	16.12	20.8		15.73	25.16	15.54	23.58
21	16.98	**End Point**		15.68	25.72	16.14	23.53
22	17.47			16.4	26.51	16.4	24.39
23	17.6			16.8	26.5	16.52	24.06
24	17.47			16.65	25.15	16.83	23.69
25	17.25			16.08	25.28	16.86	23.6
26	17.73			17.01	26.3	16.7	23.47
27	17.27			17.08	26.24	17.1	24.18
28	17.2			17.52	25.98	16.88	23.9
29	17.39			17.6	26.25	17	23.93
30	**End Point**			17.91	25.8	17.23	23.59
31				**End Point**	26.24	17.17	23.78
32					25.57	**End Point**	23.5

Continue from last page

Day after tumour injection	Fucoidan 6	Fucoidan 1
33	26.23	23.36
38	25.99	23.65
43	26.12	23.91
48	**End Point**	23.5
53		23.46
58		23.97
63		23.8
68		23.5
73		24.98
78		24.6
83		24.83
87		**End Point**